CYTOSKELETAL REGULATION BY WNT SIGNALING IN *XENOPUS* GASTRULATION MOVEMENTS

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ABBREVATIONS

Arp:	actin related protein
BCR:	blastocoel roof
DMZ:	dorsal marginel zone
Dsh:	Dishevelled
ECM:	extracellular matrix
FAK:	focal adhesion kinase
FN:	fibronectin
Fz7:	Frizzled7
G_{i1}:	$G\alpha_{i1}$
G_{11}:	$G\alpha_{11}$
GAP:	GTPase activating protein
GEF:	guanine nucleotide exchange factor
GFP:	gleen fluorescent protein
JNK:	c-jun N-terminal kinase
MARCKS:	myristoylated alanine rich c kinase substrate
MLC:	myosin light chain
MO:	morpholino oligo
PCP:	planar cell polarity
PIP2:	phosphatidylinositol 2-phosphate
PKC:	protein kinase C
PLC:	phospholipase C
PMA:	phorbol 12-myristate 13-acetate
RFP:	red fluorescent protein
RMA:	RFP-moesin actin binding domain fusion protein
SDS-PAGE:	sodium dodecyl sulfate- polyaclylamide gel electrophoreis
WASP:	Wiskott-Aldrich syndrome protein
YFP:	yellow fluorescent protein

ABSTRACT

Gastrulation is one of the most important developmental events for many multicellular organisms. In the amphibian embryos, mesodermal cells involute to the inside of the embryo and migrate along the blastocoel roof (BCR) to establish the three germ layer structure. This process involves several morphogenetic cell movements including convergent extension (Solnica-Krezel et al.,1995; Keller et al., 2000). In convergent extension, cells are polarized, elongated mediolaterally, and then intercalated each other. Wnt/PCP (Planar Cell Polarity) pathway has been implicated in the regulation of convergent extension. One of the PCP signaling components Dishevelled (Dsh) is essential for convergent extension. However, it is largely unknown how the PCP pathway regulates convergent extension.

Because active cell movements occur during convergent extension, the regulation of cytoskeletal dynamics may be important for the regulation of this process. In order to investigate the regulatory mechanisms of actin cytoskeleton during gastrulation, I cloned a gene encoding an actin-binding protein MARCKS (Myristoylated Alanine Rich C-Kinase Substrate) from a *Xenopus* embryonic cDNA library. MARCKS was first identified as a PKC (Protein Kinase C) substrate in mammalian cells. It attaches with the plasma membrane through N-terminal myristoylation. I showed that loss of MARCKS function by MO (Morpholino oligonucleotide) in *Xenopus* embryo induced a gastrulation defect phenotype without affecting mesoderm induction. To elucidate why *MARCKS* MO caused gastrulation defect, cell biological analyses were conducted. During convergent extension, *MARCKS* MO inhibited polarization and intercalation of mesodermal cells. Cell adhesion, protrusive activity and cortical actin formation were also inhibited. Furthermore, I found that activation of the PCP signaling pathway promoted formation of filopodia- and lamellipodia-like structures in ectoderm explant cells, and *MARCKS* MO inhibited this activity. These results indicate that MARCKS regulates cortical actin dynamics, and it is requisite for the morphological processes regulated by the PCP pathway. Taken together, MARCKS is an essential molecule for gastrulation movements regulated by

the PCP pathway through controlling the cortical actin formation.

It is known that Dsh is translocated to the plasma membrane in response to Wnt signaling in animal cap cells. In this thesis, I showed the bipolor localization of Dsh in mesodermal cells during convergent extension. These data indicate that the regulation of Dsh localization is important for convergent extension. But its regulatory mechanism is unknown. Thus, I analysed molecular mechanism to regulate Dsh localization and identified three proteins involved in the PCP pathway, PKC δ, Gα_{11} (G_{11}) and Gα_{i1} (G_{i1}). First I identified PKC δ as an essential factor to regulate Dsh localization and showed that it physically interacted with Dsh. Loss of PKC δ function induced a gastrulation defective phenotype without affecting mesoderm induction. Confocal microscopic analyses revealed that both PKC δ and Dsh were translocated from the cytoplasm to the plasma membrane by Fz7 signaling. In addition, loss of PKC δ function reduced the signal-dependent Dsh translocation. These results indicate that PKC δ regulates Dsh localization under the control of Wnt signaling. Next, I focused on heterotrimeric G protein α subunits. Injections of antisense MOs against G_{11} or G_{i1} caused a phenotype in the body axis elongation and/or gastrulation defect. In addition, these MOs inhibited elongation of DMZ explants. These results suggested that these G proteins might be required for convergent extension. Thus, I investigated functions of these G proteins in the PCP pathway and found that G_{i1} and G_{11} are necessary for the membrane localization of Dsh. G_{11} MO reduced both hyperphosphorylation of Dsh and the protrusive activity induced by the PCP pathway, whereas G_{i1} MO did not. These data indicate that both G_{i1} and G_{11} are required for the Dsh translocation, but these molecules may play distinct roles. These results are shown and discussed in Chapter 3.

This work demonstrates that the PCP pathway regulates convergent extension movements through cytoskeletal regulation, and identified molecules essential for the intracellular signaling components in this pathway. These findings may contribute to understand the mechanisms of convergent extension movements and the other developmental processes in which the PCP pathway is involved.

CHAPTER 1

GENERAL INTRODUCTION

During embryogenesis and organogenesis, polarity, shape, and migration of each cell must be carefully regulated. Secreted protein factors including Wnts have been shown to play important roles in such morphogenetic processes. These molecules regulate such processes not only through transcriptional activities, but also through effector molecules directly linked to cytosleletal dynamics. During *Xenopus* gastrulation, embryonic tissues are greatly rearranged and reorganized. Particularly, dorsal mesodermal cells show active migration with dynamic change of their cell shape, providing me an attractive model system to study the regulatory mechanisms of cell polarity, morphology and movement at the cellular level.

During gastrulation, mesodermal cells migrate to the inside of the embryo and move along the blastocoel roof (Fig. 1-1). This movement is essential for the establishment of the three germ layers and body axes. The process involves highly integrated cell movements. One of the important mechanisms for this movement is convergent extension (Fig. 1-1). As convergent extension begins, cells are polarized and aligned mediolaterally; this is followed by the intercalation of these polarized cells. This movement elongates the mesodermal tissue along the anteroposterior axis, producing a driving force for gastrulation movements (Wilson and Keller 1991; Shih and Keller 1992; Wallingford et al., 2002).

Regulation of convergent extension movements is known to involve Wnt signaling pathways. Wnts are a family of secreted proteins that regulate many biological processes (Cadigan and Nusse 1997). Functional analyses in *Xenopus* suggest that the Wnt family can be divided into two functionally distinct groups. The first group of Wnts induces a secondary axis when ectopically expressed in *Xenopus* embryos. They activate the canonical Wnt/β-catenin pathway and induce transcription of target genes such as *siamois* and *Xnr3* (Brannon and Kimelman 1996; Carnac et al., 1996;

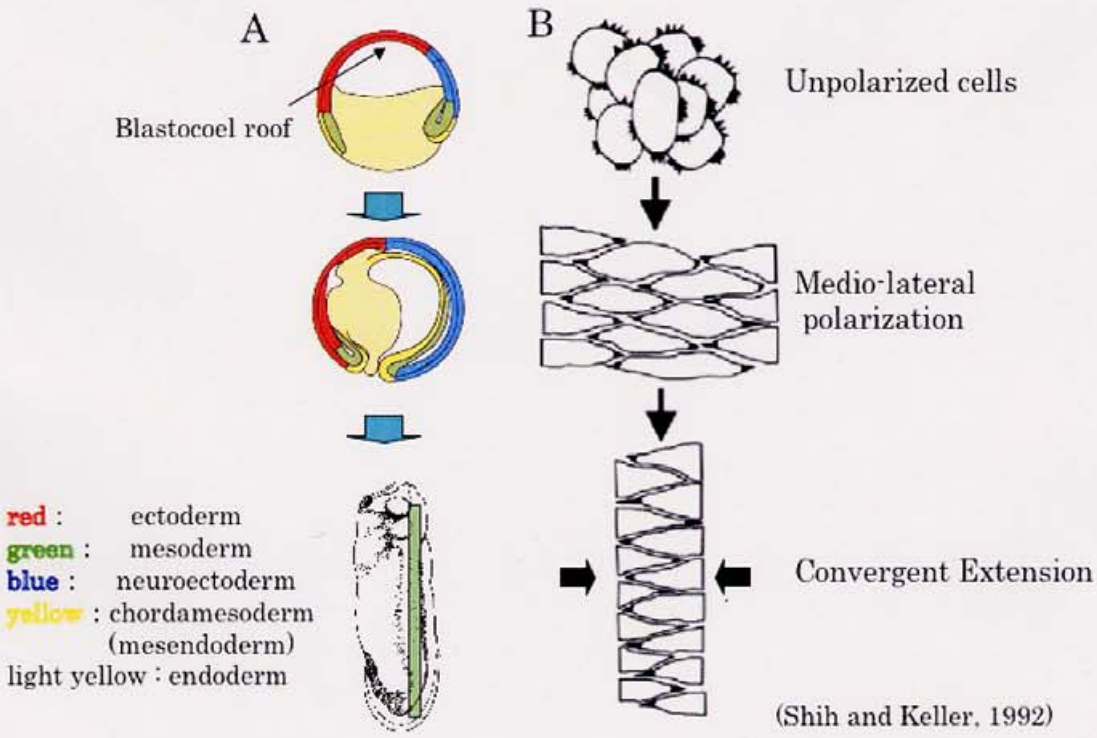

Figure 1-1. Gastrulation movements and convergent extension.

(A) During gastrulation, mesoderm involutes into the embryo, and elongate along the blastocoel roof. (B) In this movement, mesodermal cells are polarized and intercalated mediolaterally, and elongate the tissue along the anteroposterior axis

McKendry et al., 1997). The second group of Wnts, which include Wnt5a and Wnt11, activates the noncanonical Wnt signaling pathway that controls morphogenetic cell movements (Kuhl et al., 2002; Tada et al., 2002). The noncanonical Wnt pathway branches into two cascades. One is the PCP pathway, and the other is the Wnt/Ca^{2+} pathway (Kuhl et al., 2000). In *Drosophila*, the PCP pathway specifies cell polarities in epithelial cells and other types of cells (Adler et al., 2002). Accumulating evidence shows that the PCP pathway also plays crucial roles to regulate cell polarity and morphogenesis in vertebrates. Convergent extension is known as one of the morphogenetic movements regulated by PCP signaling in *Xenopus* and zebrafish. Although extensive studies on the PCP pathway have been carried out and identified signaling molecules in this pathway, including Rho GTPases and JNK (c-Jun N-terminal Kinase), it is still largely unknown how this pathway regulates convergent extension.

Cell biological analyses on convergent extension movements have been performed by several groups. Harland and his colleagues have found that dorsal marginal zone (DMZ) cells form lamellipodia in the mediolateral direction in the *Xenopus* embryo. At the same time, they showed that overexpression of wild type or dominant negative mutant of Dsh randomized the lamellipodial orientation. They concluded that Dsh controls cell polarity and orientation of cell migration (Wallingford et al., 2000). Heisenberg's group analyzed zebrafish Wnt11 mutant called *silberblick* (*slb*), and found that convergent extension was inhibited in the mutant embryos. Furthermore, wild type prechordal plate precursor cells form pseudopods to the migrating orientation, whereas those cells in *slb* mutant showed the formation of randomly oriented pseudopods (Heisenberg et al., 2003). These reports suggest that the PCP pathway regulates cell migration and cell shape change through the regulation of actin cytoskeleton.

Rho family GTPases including RhoA, Cdc42, and Rac play critical roles in the regulation of actin cytoskeleton. The importance of Rho family in the PCP pathway has also been demonstrated (Fig. 1-2). Winterd et al. (2001) showed that *Drosophila* Rho kinase (Drok) mutant displayed the randomly oriented wing hair and photoreceptor cells and concluded that

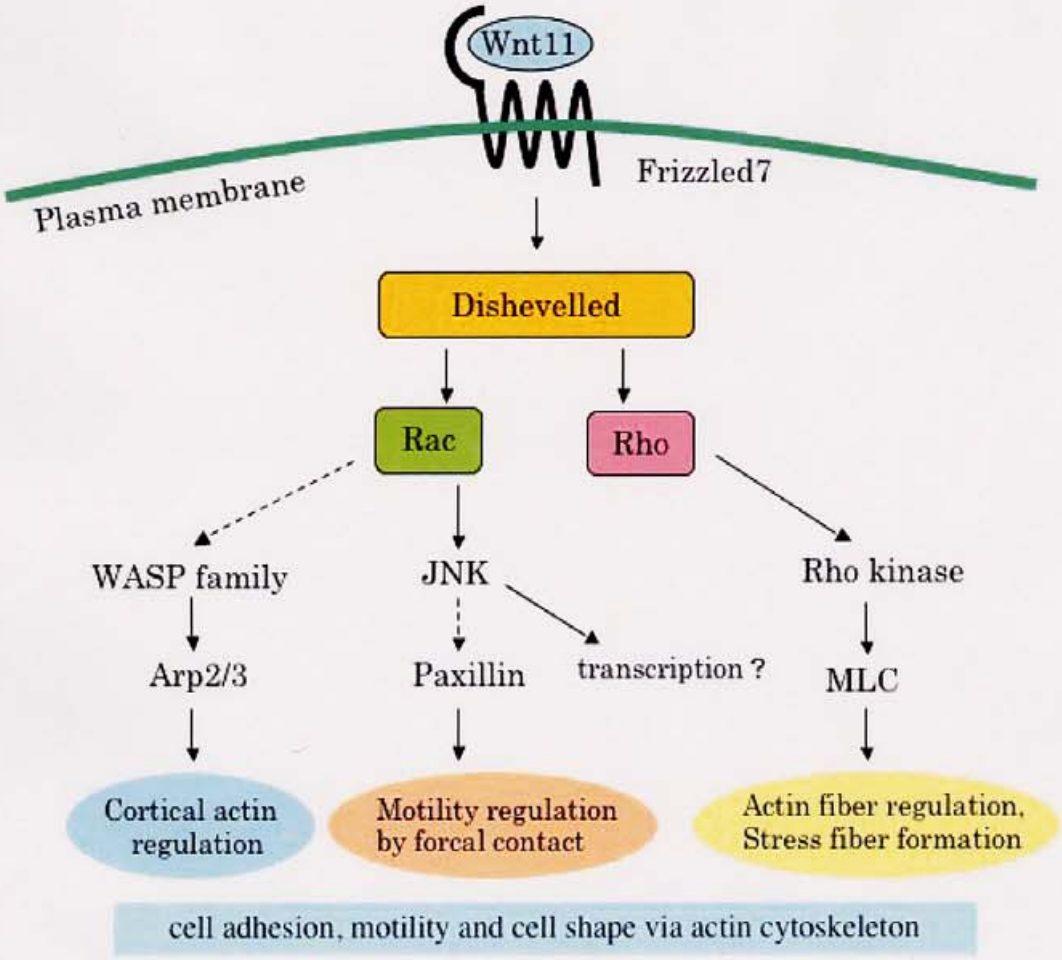

Figure 1-2. The PCP signaling pathway and the effector proteins in vertebrates.

Wnt signaling activates Dsh through the Fz7, and Dsh activates Rho family small GTPases. Regulatory molecules (ex. guanine nucleotide exchange factors (GEF), GTPase activating proteins (GAP)) may regulate these GTPase activity. But the relationship between Dsh and these regulatory molecules is unclear. The pathway represented by dotted lines are unclear in the PCP pathway.

Drok was essential for epidermal planar cell polarity. They also showed that the multiple hair phenotype caused by *fz* or *dsh* mutant is rescued by overexpression of Drok. This genetic interaction suggests that Drok functions in the downstream of Fz and Dsh. This is consistent with the result that *slb* phenotype is rescued by overexpression of Rho kinase2 in zebrafish (Marlow et al., 2002). Upon the activation of the Wnt/Fz pathway, Dsh forms a complex with RhoA and Rac in the *Xenopus* embryo (Habas et al., 2003). This complex formation is important for the regulation of convergent extension. In addition, it is also known that Cdc42 regulate cell adhesion via Wnt/Ca^{2+} pathway during *Xenopus* gastrulation (Choi et al., 2002). Although several proteins homologous to *Drosophila* PCP components regulate the convergent extension movements in vertebrates, the signaling pathway may not be identical with the *Drosophila* PCP pathway. For example, *Xenopus* PCP pathway activates JNK via Rac in contrast to *Drosophila* in which the PCP pathway activates JNK via RhoA (Axelrod et al., 2000).

JNK is also an important factor for cytoskeletal regulation of PCP pathway. It has been shown that ectopic Wnt5a or Dsh expression induce JNK activation, and JNK function is essential for convergent extension movements (Yamanaka et al., 2002). However, it is unclear how JNK is involved in the regulation of convergent extension through the PCP pathway. JNK has been shown to play a key role in cell motility in other systems. For example, JNK phosphorylates one of the focal complex component, paxillin, and regulates cell adhesion and motility (Huang et al., 2003). The link between the PCP pathway and cell adhesion has also been suggested in the *Xenopus* embryos (Marsden et al., 2001). FN (fibronectin) is known as an extracellular matrix protein that interacts with integrins. FN fibrils cover the surface of the BCR on which the mesodermal cells crawl during gastrulation. It has been shown that FN and integrins play important roles in Dsh localization in the mesodermal cells during gastrulation. Wnt signaling may crosstalk with the integrin/FN signaling.

The main purpose of this study is to elucidate (i) the mechanisms of cytoskeletal regulation by PCP signaling during convergent extension, and (ii) the mechanism of signal transduction in the PCP pathway. In Chapter 2,

I focused on the function of actin binding protein MARCKS. Overexpression of MARCKS in DMZ caused gastrulation defects. By visualizing the cell shape, motility and molecular localization, I demonstrated the importance of MARCKS in PCP signaling during *Xenopus* convergent extension movements. In Chapter 3, I describe the analyses of the PCP signal transduction mechanism. It is known that Dsh is translocated from the cytoplasm to the plasma membrane upon the Wnt signal activation. I found that three molecules, PKC δ, G_{i1}, and G_{11} were essential for the Dsh translocation and for convergent extension.

These findings may contribute at least in part, to provide answers to following questions : (1) What is the role of the PCP pathway in the regulation of convergent extension, and (2) How is the PCP pathway activated.

CHAPTER 2

"The actin binding protein MARCKS is essential for *Xenopus* convergent extension movements"

SUMMARY

MARCKS is an actin-binding, membrane-associated protein expressed during *Xenopus* embryogenesis. I analyzed its function in cytoskeletal regulation during gastrulation. Here, I show that blockade of its function impaired morphogenetic movements, including convergent extension. MARCKS was required for the control of cell morphology, motility, adhesion, protrusive activity, and cortical actin formation in embryonic cells. I also demonstrated that the PCP pathway promotes the formation of lamellipodia- and filopodia-like protrusions and that MARCKS is necessary for this activity. These findings show that MARCKS regulates the cortical actin formation that is requisite for the dynamic morphogenetic movements during gastrulation.

INTRODUCTION

When cells migrate in contact with other cells, extracellular matrices, or other substrates, cells forms protrusions such as filopodia, lamellipodia and pseudopods towards the migrating orientation. This activity is based on the actin cytoskeletal dynamics. Actin filaments are formed and elongated by the assembly of monomeric globular actin. In addition, actin filaments are bundled, severed, or branched to form mesh like structures. These dynamic aspects of actin filament produce driving force for the protrusive activity and cell migration. Many regulatory proteins participate in the regulation of this actin dynamics.

Because convergent extension is accompanied by dynamic changes in cell polarity, morphology, and motility, it is very likely that cytoskeletal dynamics are carefully regulated. Although the PCP pathway has been shown to be essential for the regulation of this process, the relationship between the PCP pathway and actin cytoskeleton is poorly understood. Thus, I sought to analyze the regulatory mechanism of actin dynamics during gastrulation and elucidate how the PCP pathway is involved in the regulation of actin cytoskeleton.

I decided to focus on myristoylated alanine-rich C kinase substrate (MARCKS). Mammalian MARCKS has been shown to interact with actin filaments (Arbuzova et al., 2002). It has been reported that *Xenopus MARCKS* is expressed maternally and throughout embryogenesis (Ali et al., 1997; Shi et al., 1997), but its role in development was not well unknown.
Here, I report that the loss of MARCKS function severely impaired gastrulation movements. MARCKS regulates the cortical actin formation, cell adhesion, protrusive activity, and cell polarity control during gastrulation. I further show that MARCKS is necessary for the protrusive activity regulated by the PCP pathway. These findings indicate that MARCKS regulates the cortical actin formation that is requisite for dynamic morphogenetic movements.

EXPERIMENTAL PROCEDURES

Plasmids, RNA synthesis, and Morpholino oligos

Procedures for the plasmid construction, RNA synthesis and sequences of Morpholino oligos were described in the online supplemental material. The RFP plasmid is a gift from R. Tsien (University of California, San Diego,CA).

In situ hybridization and RT-PCR analysis

In situ hybridization in *Xenopus* was performed as described by Harland (1991). For RT-PCR analyses, RNA from the explants was prepared with TRIzol (Life Technologies). cDNA was synthesized with reverse transcriptase (TRT-101; Toyobo). Sequences of the primers were described in the online supplemental material.

Whole-mount immunostaining and Western blotting

The procedure for whole-mount immunostaining was performed as described in Kurata et al. (2001). The antibodies were MZ15 for notochord (a gift from F. Watt, Imperial Cancer Research Fund, London, UK) and 12/101 for somites (Development Studies Hybridoma Bank). Western blotting was performed using a mouse monoclonal anti-pan-actin antibody was purchased from NeoMarkers (MS-1295-P0).

Dissecting explants and cytological observations

For the animal cap explants, *MARCKS* mRNA or MO was coinjected with 0.5 pg *activin* mRNA into the animal pole of two-cell embryos. The animal cap was dissected from stage 9 embryos. For DMZ explants, mRNA or a MO was injected into the two dorsal blastomeres of four-cell embryos. Explants were isolated at stage 10.5. These explants were cultured in 1x Steinberg's solution until sibling embryos reached stage 17. To dissociate cells from the explants, the explants were incubated in the Ca^{2+}-Mg^{2+}-free medium for 2 h. For the cytological observation, explants and dissociated cells were cultured in 1x Steinberg's solution on an FN-coated dish

(4000–030; Iwaki), or on a cover glass coated with FN (2 μ g/cm2, F1141; Sigma-Aldrich). To stain F-actin, cells were fixed in 4% PFA and stained with PBS 0.5% Triton X-100 containing a 40-fold dilution of BODIPY 581/589 phalloidin (B-3416; Molecular Probes) or Alexa Fluor 488 phalloidin (A-12379; Molecular Probes). For confocal microscopy, images were captured using 510 software (Carl Zeiss MicroImaging, Inc.). All images were prepared for publication using Adobe Photoshop software.

RESULTS

Expression pattern of *MARCKS* in *Xenopus* embryos

First, I examined the expression pattern of *MARCKS* in early *Xenopus* embryos. It has previously been shown that *MARCKS* is maternally expressed, and zygotic expression is started before gastrulation (Shi et al., 1997). I showed that *MARCKS* was ubiquitously expressed in the gastrula and specifically expressed in the head and the neural tube in the neurula embryos (Fig. 2-1).

MARCKS is required for normal development

To investigate the function of MARCKS in *Xenopus* development, I carried out loss of function experiments using antisense MO. At first, I examined the specificity of *MARCKS* MO. The MO specifically and effectively inhibited epitope-tagged MARCKS protein synthesis, leading us to expect that it could inhibit the endogenous MARCKS protein synthesis (Fig. 2-2 A).

Using *MARCKS* MO, I analyzed MARCKS function in development. When it was injected into the dorsal marginal zone (DMZ) of four-cell embryos, the embryos showed a gastrulation-defective phenotype (Fig. 2-2 B). The effect was dose-dependent. The involution of the mesoderm was impaired and the blastopore remained open. A similar phenotype was observed when *MARCKS* mRNA was injected. The phenotype of *MARCKS* MO was partially rescued by coinjection of *MARCKS* mRNA. The rescue was imperfect (Fig. 2-2 C), probably because both over- and under- expression of *MARCKS* have negative influence on gastrulation movements. MARCKS is essential for gastrulation and its level must be tightly regulated.

MARCKS is not required for mesodermal induction

Next, to determine whether the gastrulation defect caused by loss of MARCKS function was due to the defect in mesodermal differentiation, I examined the expression of the dorsal mesodermal markers. At the gastrula stage, *MARCKS* MO-injected embryos expressed *chordin* at the same level a

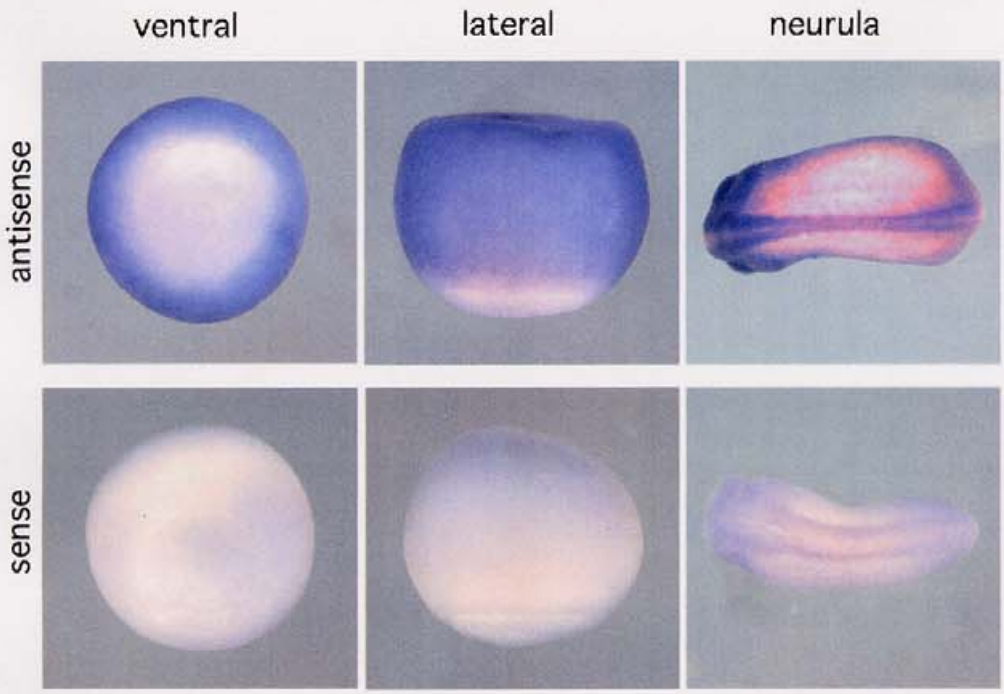

Figure 2-1. *in situ* hybridization probed with *MARCKS*.

MARCKS is ubiquitously expressed in the early gasturula embryo. The head and the neural tube specific expression was observed in the neurula embryos.

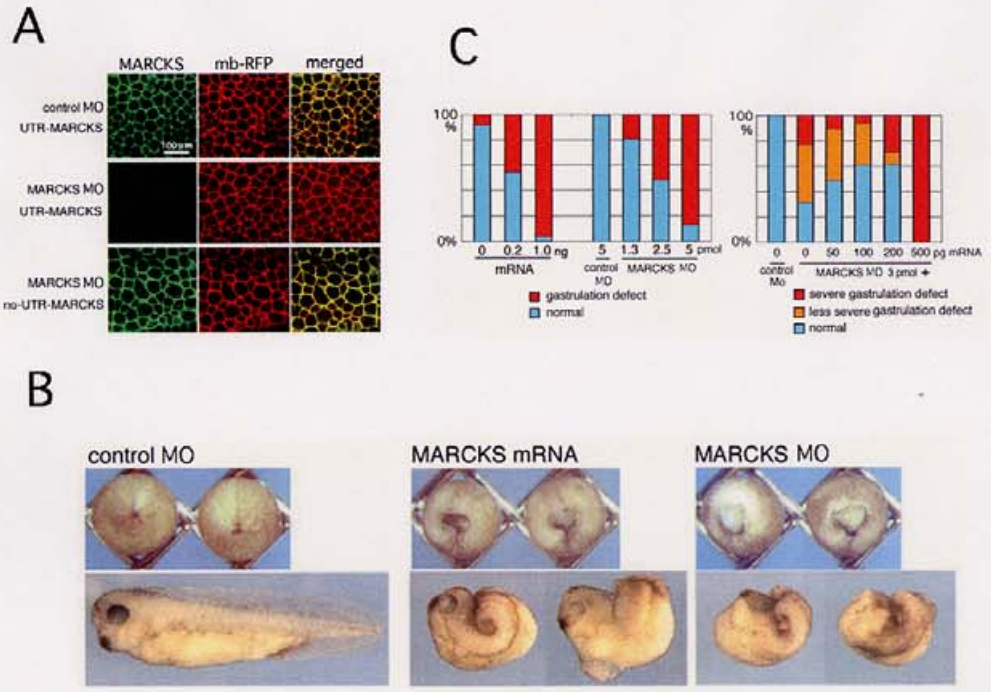

Figure 2-2. MARCKS is essential for convergent extension.

(A) *MARCKS* MO specifically inhibited MARCKS protein synthesis. *MARCKS-Venus* fusion genes with or without the 5'-untranslated region (UTR) containing the Mo-targeting sequence were constructed (*UTR-MARCKS* and *no-UTR-MARCKS*, respectively). When they were expressed in animal cap explants, both *UTR-* and *no-UTR-MARCKS* were detected at the same levels and were located on the plasma membrane. *MARCKS* MO inhibited the translation of *UTR-MARCKS*, but not of *no-UTR-MARCKS*. The protein expression of an unrelated control, membrane-binding red fluorescent protein (Campbell et al., 2001) (mb-RFP), was not affected. This result led us to expect that *MARCKS* MO could specifically and effectively inhibit the endogenous MARCKS protein synthesis.(B) Both 500 pg of *MARCKS* mRNA and 5 pmol of *MARCKS* MO impaired gastrulation movements, when either was injected into the dorsal marginal region. (C) Statistical data of the gastrulation-defective phenotype caused by *MARCKS* mRNA and MO.

control embryos (Fig. 2-3 A). In tadpoles, the notochord and somites were formed in the *MARCKS* MO injected embryos, but the extension of these tissues was severely inhibited (Fig. 2-3 B). I also tested the expression of the mesodermal markers in DMZ explants by RT-PCR (Fig. 2-3 C). The expression of these markers was not inhibited by *MARCKS* MO. These results indicated that the phenotype was caused, not by a defect in mesoderm differentiation, but by a defect in morphogenetic movements.

MARCKS is essential for gastrulation movements

Next, I tested whether the loss of MARCKS function affects the animal cap elongation, which mimics convergent extension movements during gastrulation (Fig. 2-4 A). *MARCKS* MO blocked the elongation by activin, and it was rescued by coinjecting *MARCKS* mRNA without containing *MARCKS* MO target site, suggesting that MARCKS is required for convergent extension. During mesodermal convergent extension, the cells become polarized, align mediolaterally, and are then intercalated. To test how MARCKS is involved in this process, the convergent extension in DMZ explants was observed microscopically. *MARCKS* MO, Rhodamine dextran, and mRNA encoding *membrane-binding Venus* (*mb-Venus*) were coinjected into one of the two dorsal blastomeres (Fig. 2-4 B). As a control, *mb-Venus* mRNA alone was injected into the other dorsal blastomere. At the gastrula stage, the DMZ explants were isolated and cultured on a cover glass coated with FN. These explants adhered to the FN, and convergent extension movements occurred subsequently in the mesoderm (Kinoshita et al., 2003). In the absence of *MARCKS* MO, red and non-red cells were polarized and intercalated. In the *MARCKS* MO-injected explants, the non-red cells, which were assumed to lack the MO, were polarized and showed convergent extension. In contrast, the red cells (MO-injected cells) were not polarized and did not participate in the intercalation. Thus, MARCKS is essential for the cell polarization and movement during convergent extension.

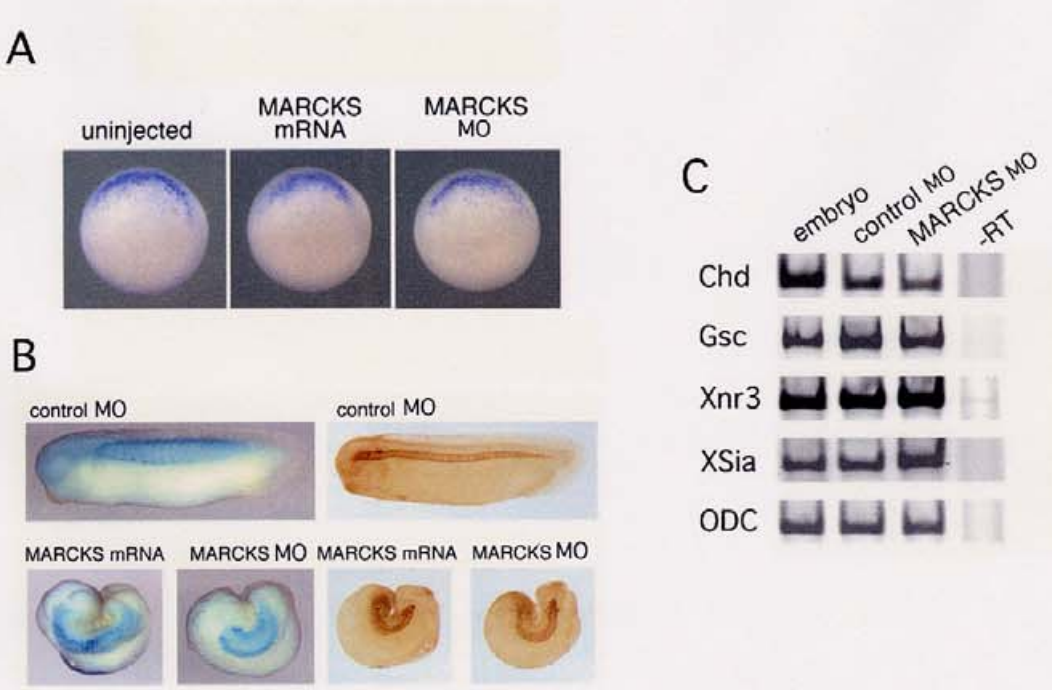

Figure 2-3. MARCKS in not required for the induction of mesodermal marker genes.

(A) Expression of *chordin* at the gastrula stage, detected by in situ hybridization. (B) Somites (left) and notochord (right) were immunostained with 12/101 and MZ15 antibodies, respectively. (C) 5 pmol of *MARCKS* MO was injected into the two dorsal blastomeres at the four-cell stage; the DMZ explants were isolated, and the expression of mesodermal markers was detected by RT-PCR. gsc, *goosecoid*.

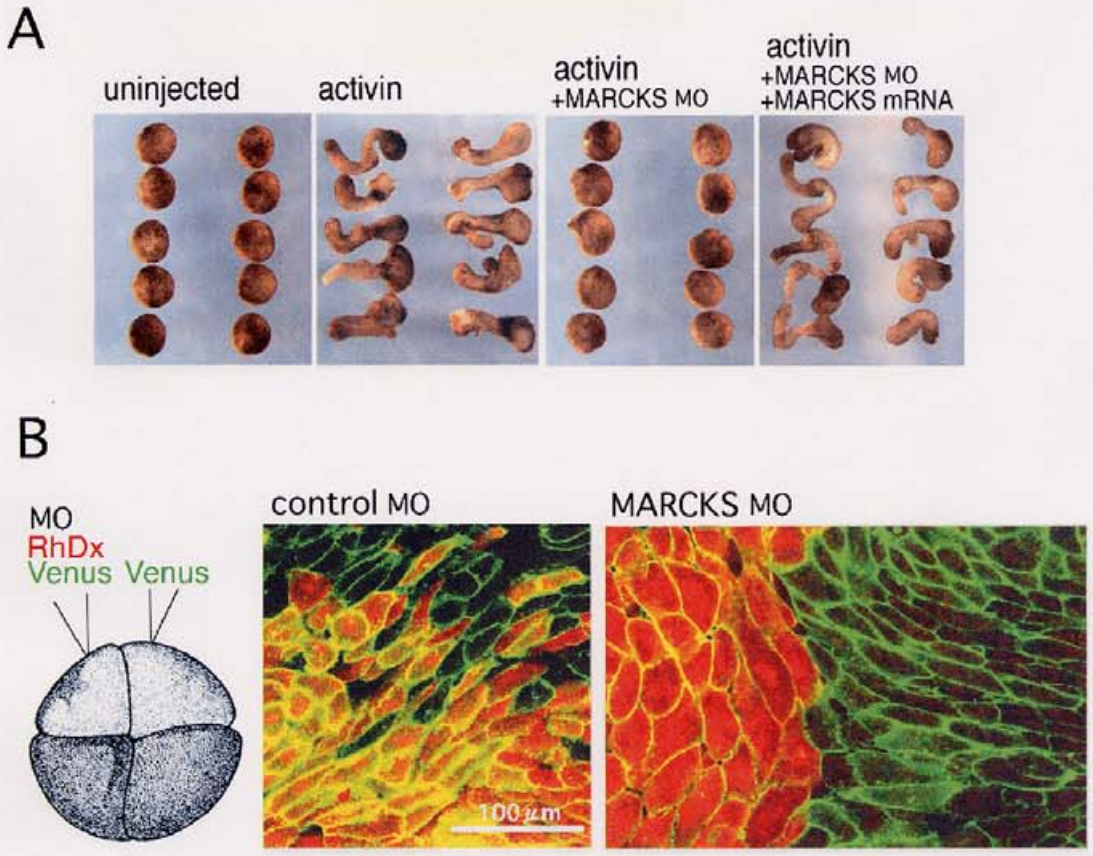

Figure 2-4. MARCKS is essential for convergent extension.

(A) 2 pmol of *MARCKS* MO inhibited the *activin* mRNA-induced elongation of animal caps. This inhibition was rescued by coinjection of 200 pg of *MARCKS* mRNA. (B) 5 pmol of *MARCKS* MO, Rhodamine dextran, and the mRNA for 100 pg of *mb-Venus* were coinjected into one of the two dorsal blastomeres at the four-cell stage. *mb-Venus* mRNA alone was injected into the other dorsal blastomere. DMZ explants were cultured on a cover glass coated with FN, and convergent extension movements were observed.

MARCKS function is required for mesendodermal cell migration

In addition to convergent extension, an important mechanism regulating gastrulation movements is mesendoderm extension (Davidson et al., 2002). To test whether MARCKS is required for this process, DMZ explants were cultured on FN-coated dishes according to the method developed by Davidson et al. (2002). Mesendodermal cells migrated on the FN substrate as an intact mantle (Fig. 2-5). When *Venus* mRNA and the control MO were coinjected, the Venus expressing cells dispersed broadly, and some cells migrated to the front. In contrast, *MARCKS* MO-injected cells rarely migrated on the FN substrate. I examined 15 explants and confirmed that none of the *MARCKS* MO-injected cells reached the leading edge of the migrating mesendoderm. This indicated that MARCKS was required for mesendoderm extension as well as convergent extension.

MARCKS is essential for cell adhesion and spreading on FN

I examined whether *MARCKS* MO affects the adhesion to FN of cells dissociated from the DMZ explants. *MARCKS* MO and *Venus* mRNA (green) or Rhodamine dextran (red) was injected dorsally. DMZ explants were isolated and dissociated in Ca^{2+}-Mg^{2+}-free medium. Dissociated cells were cultured on FN-coated dishes, and cells that adhered to the dish were counted (Fig. 2-6). When *MARCKS* MO was coinjected with Venus, the adherence of Venus-expressing cells was extremely reduced. In contrast, when *MARCKS* MO was coinjected with Rhodamine dextran, these red cells rarely adhered to the dish. A few cells containing *MARCKS* MO were found on the dish, but these cells were rounded up and did not spread out on the dish. This indicates that MARCKS is essential for cell adhesion and spreading on FN.

Protrusive activity of mesodermal cells reduced by loss of MARCKS function

Next, I tested whether *MARCKS* MO affected the protrusive activity in mesodermal cells. Mesodermal cells had many filopodia-like protrusions when the DMZ explants adhered to a FN-coated dish. *MARCKS*

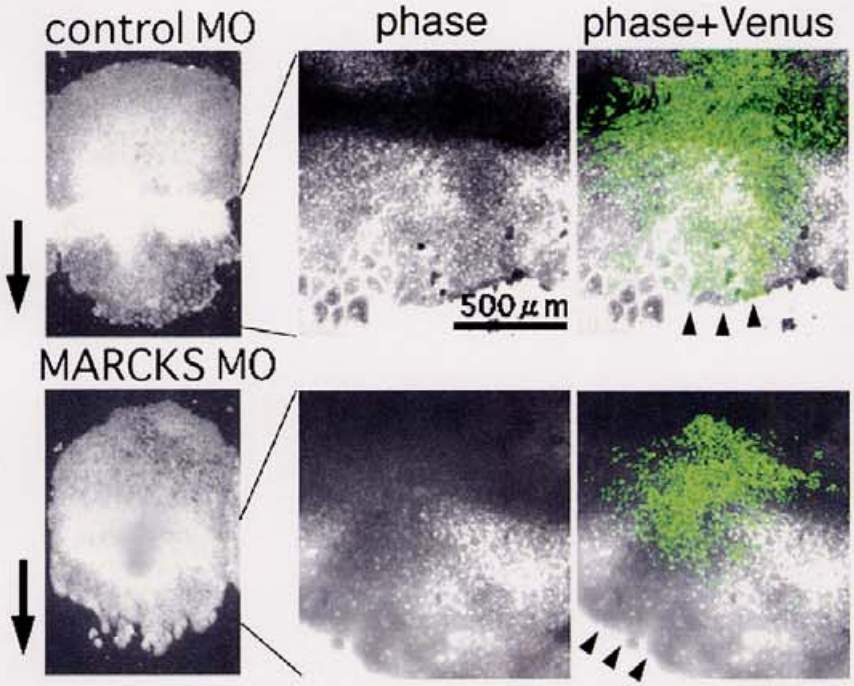

Figure 2-5. MARCKS is required for mesendodermal cell migration.

Control or 5 pmol of *MARCKS* MO was coinjected with 100 pg of *Venus* mRNA into two blastomeres of four-cell embryos. DMZ explants were cultured on an FN-coated dish until sibling embryos reached the late neurula stage. Arrows indicate the direction of mesendoderm migration. Arrowheads indicate the leading edge.

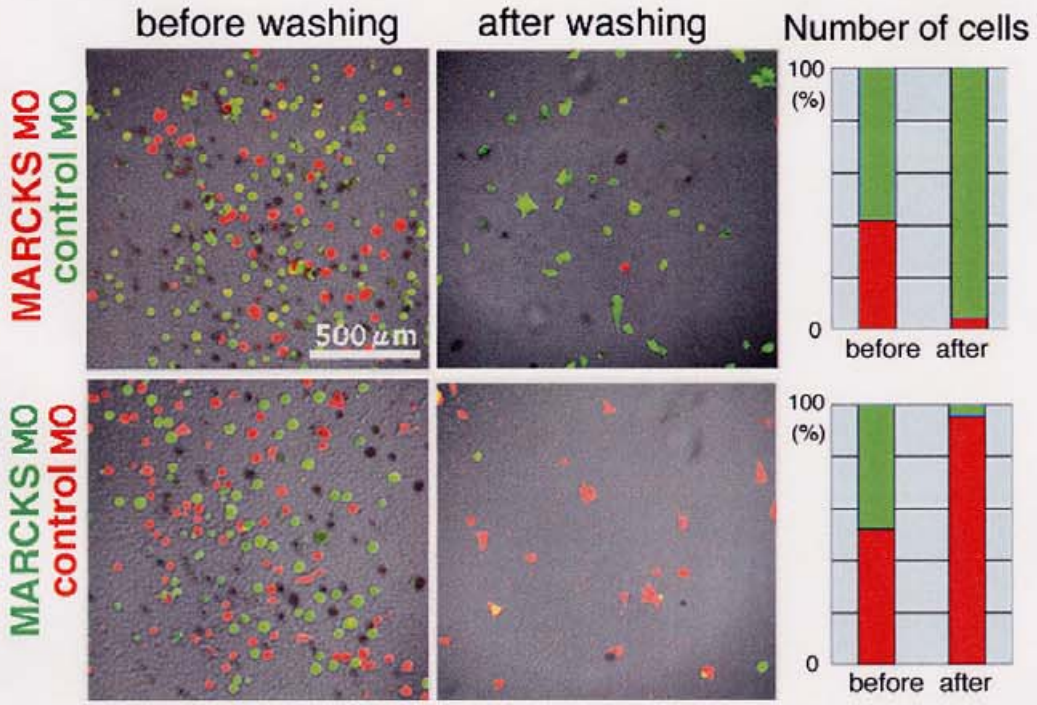

Figure 2-6. MARCKS is essential for cell adhesion and spreading on FN

MARCKS MO inhibited the adhesion on FN. *MARCKS* MO, control MO, *Venus* mRNAs (green), and Rhodamine dextran (red) were coinjected dorsally as indicated. Cells were dissociated from the DMZ explants. Cells from the control- and *MARCKS* MO–injected explants were mixed, plated on FN-coated dishes, incubated for 6 h, and fixed in formaldehyde. Cells that did not adhere to the dish were removed by washing five times with PBS.

MO severely reduced the number and the length of these protrusions (Fig. 2-7). The effect of *MARCKS* MO on the protrusive activity was rescued by coinjection with *MARCKS* mRNA. Thus, MARCKS is required for the protrusive activity, which may directly correlate with the control of cell adhesion and motility. The inhibition of cell adhesion and migration on the FN fibrils that cover the blastocoel roof may contribute to the gastrulation defect caused by *MARCKS* MO.

Subcellular MARCKS localization may be regulated by phosphorylation of actin binding domain.

At first, I examined the colocalization of MARCKS with F-actin. Cells expressing *MARCKS-Venus* were dissociated from DMZ explants and cultured on an FN-coated dish. The cells were then fixed and F-actin was stained with phalloidin. As shown in Fig. 2-8, MARCKS and cortical actin were colocalized. I then constructed two mutants, GA and SD (Fig. 2-10). GA is an unmyristoylated mutant in which the second glycine residue is replaced with alanine. SD is a pseudophosphorylation mutant whose potential phosphorylation sites were replaced with aspartic acid, which is expected not to bind to actin filaments (Hartwig et al., 1992). To detect F-actin, I used the F-actin–binding domain of moesin fused to red fluorescent protein (RFP; Campbell et al., 2002), designated RMA (RFP-moesin actin–binding domain). It has been shown biochemically that this domain binds to F-actin (Turunen et al., 1994; Pestonjamasp et al., 1995). In *Drosophila* embryos, the corresponding domain of moesin fused with GFP was successfully used to analyze actin dynamics (Dutta et al., 2002). I confirmed that our construct (Venus-moesin actin-binding domain) colocalized with stress fibers and cortical actin stained with phalloidin in CHO cells (Fig. 2-9). In *Xenopus* embryonic cells, the RMA was localized to the cell cortices, and cytochalasin B treatment, which disrupts actin filaments, dispersed the RMA to the cytoplasm (Fig. 2-10). This indicates that the RMA should be useful for monitoring F-actin dynamics. When these *MARCKS-Venus* genes were expressed, the wild-type and GA forms were associated with the plasma membrane and colocalized with RMA, but SD was in the cytoplasm. When

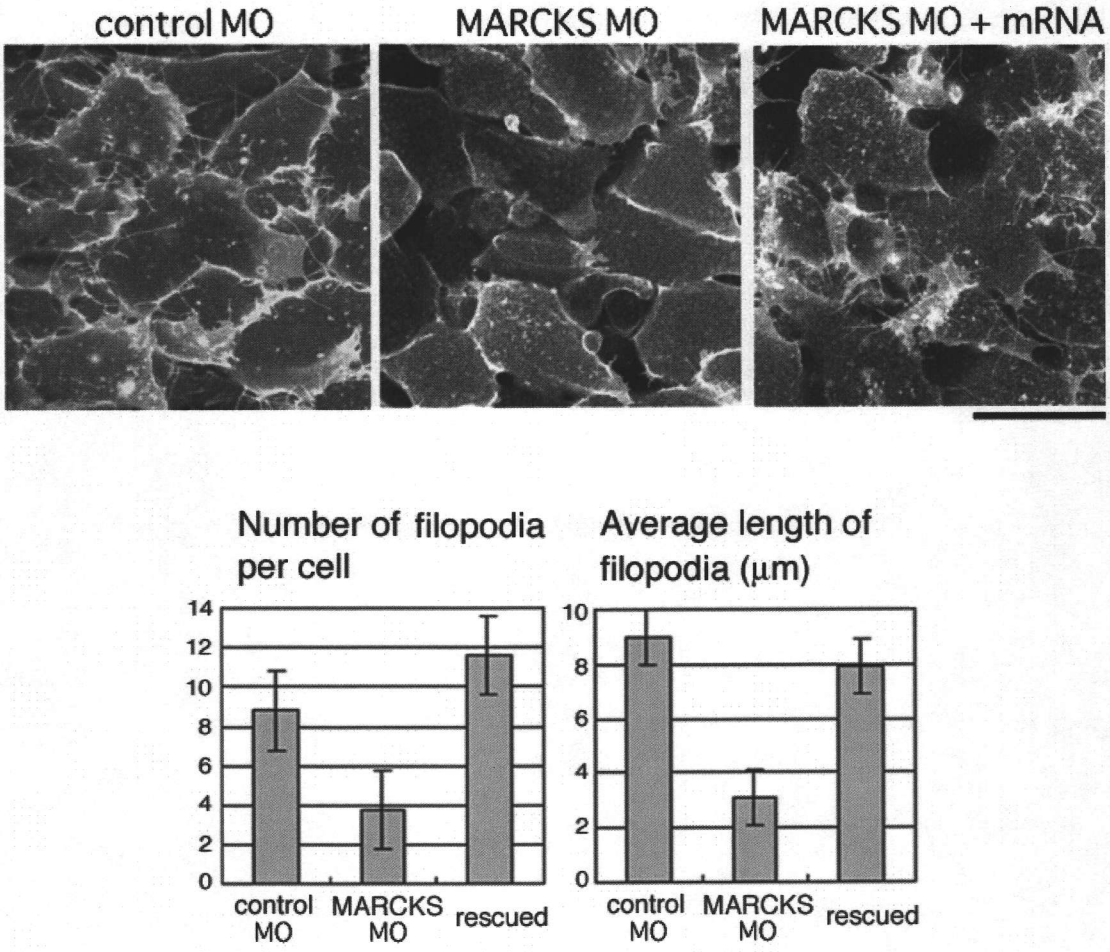

Figure 2-7. Protrusive activity of mesodermal cells reduced by loss of MARCKS function.

3 pmol of *MARCKS* MO inhibited the protrusive activity of cells in DMZ explants. *MARCKS* MO or control MO was coinjected dorsally with *mb-Venus* mRNA. DMZ explants were cultured on an FN-coated dish until sibling embryos reached the early neurula stage. The effect of *MARCKS* MO was rescued by 200 pg of *MARCKS* mRNA. Bar, 50 μm. The graph shows statistical data obtained by analyzing 15 cells for each sample. The error bars represent statistical significance (p 0.05).

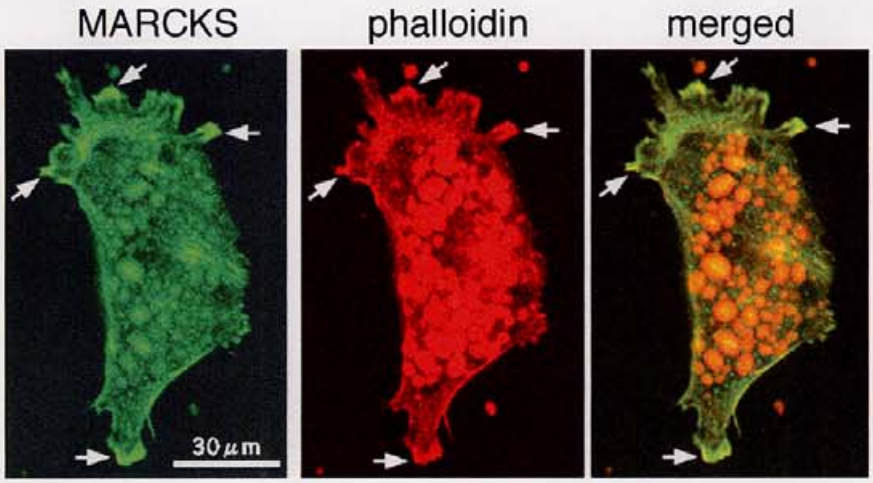

Figure 2-8. MARCKS and cortical actin were colocalized in DMZ cells.

Cells expressing 200 pg of *MARCKS-Venus* mRNA were dissociated from DMZ explants and plated on a FN-coated cover glass. Arrows indicate protrusions where both MARCKS and F-actin were enriched.

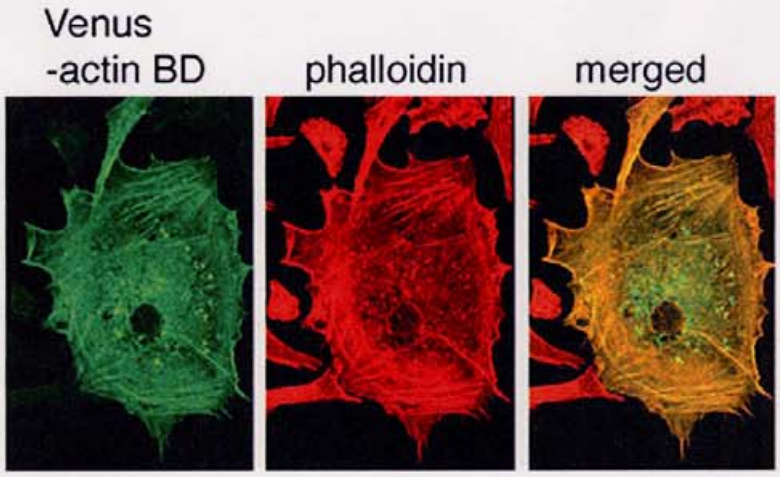

Figure 2-9. The actin binding domain of moesin is available for an indicator of F-actin in living cells.

The actin-binding domain of *Xenopus* moesin was fused with Venus (Venus-actin BD) and expressed in Chinese hamster ovary (CHO) cells.

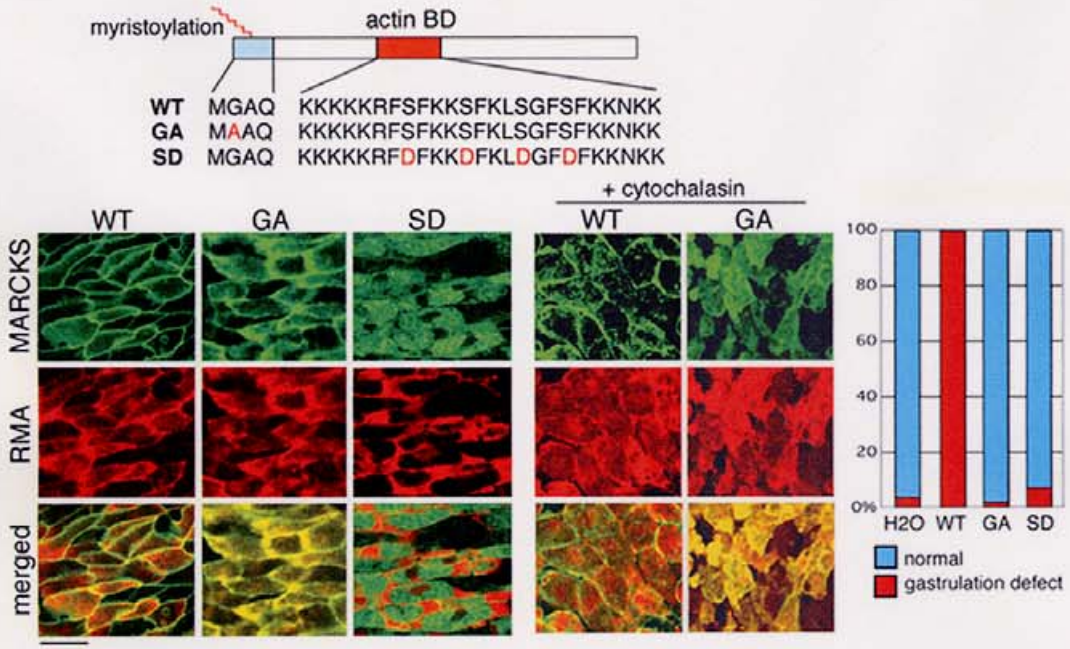

Figure 2-10. Subcellular localization of MARCKS may be regulated by phosphorylation of the actin binding domain.

Wild-type, GA, and SD mutants were expressed in the DMZ explants and observed. RMA, RFP fused with the actin-binding domain of moesin. The explants were treated with 200 μ M of cytochalasin B for 30 min. Bar, 50 μ m. The graph shows percentages of the gastrulation-defective phenotype.

the cells were treated with cytochalasin B, GA dispersed to the cytosol with RMA, whereas wild-type remained on the membrane. This result suggested that the association of MARCKS with the membrane was regulated by two mechanisms, myristoylation and binding to the cortical actin. I also found that GA and SD mutants did not inhibit gastrulation movement when they were overexpressed (Fig. 2-10). These mutants also did not rescue the embryo phenotype caused by *MARCKS* MO. These results suggest that both myristoylation and actin biding are required for its function.

MARCKS function is essential for cortical actin formation

To test whether MARCKS regulates cortical actin formation, *MARCKS* MO was injected into one blastomere near the animal pole of two-cell embryos. Animal caps were isolated at the late blastula stage, fixed, and stained with phalloidin. Membrane-binding RFP was coinjected with MO for tracing the injected cells. As shown in Fig. 2-11A, *MARCKS* MO significantly reduced the amount of cortical actin stained by phalloidin. The amount of actin protein was not affected, however, judging from Western blotting and immunocytochemistry using an anti-pan actin antibody (Fig. 2-11, B and C). This result suggests that MARCKS plays an important role in cortical actin formation.

Dsh colocalized with cortical actin in the dorsal marginal zone cells

To investigate the relationship between the Wnt pathway and cortical actin, I examined the localization of Dsh. Cells were dissociated from the DMZ explants expressing *Dsh-Venus*, cultured on an FN-coated dish, and stained with phalloidin. As shown in Fig. 2-12 A, Dsh was colocalized with cortical actin, even in the lamellipodial and filopodial protrusions. When RMA was expressed during convergent extension, it was located at the tips of elongated mesodermal cells (Fig. 2-12 B). This indicates that F-actin is enriched in this region I showed previously that Dsh-Venus was also accumulated in the same region (Kinoshita et al., 2003). Mammalian Dishevelled interacts with actin filament through the NH2 -terminal DIX

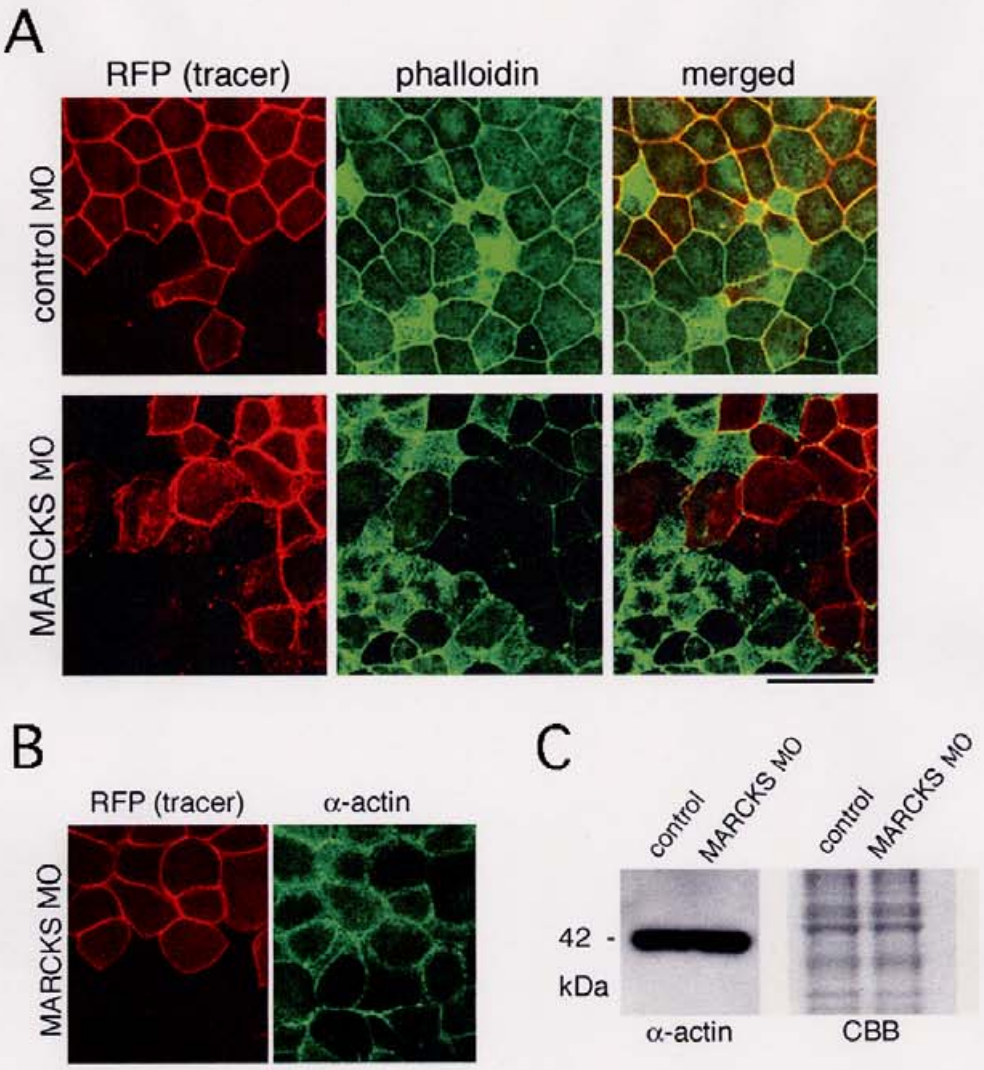

Figure 2-11. MARCKS function is essential for cortical actin formation.
(A) MO was injected into one blastomere of two-cell embryo with *mb-RFP* mRNA as a tracer. Animal cap explants were fixed and stained with phalloidin. Bar, 50 μm. (B) *MARCKS* MO and mb-RFP were coinjected and animal cap cells were immunostained with anti-actin antibody. (C) Western blot with an anti-actin antibody (left) and Coomassie Blue (CBB) staining (right). MO was injected into both of the blastomeres of two-cell embryos. Lysates were prepared from the animal caps.

domain (Capelluto et al., 2002). To test whether the tip localization of Dsh was due to the interaction between the DIX domain and F-actin, I tested the localization of Dsh lacking the DIX-domain (Dsh Δ DIX). As shown in Fig. 2-12 B, the Dsh Δ DIX was located at the tip, indicating that this localization is not due to interaction between F-actin and the DIX domain. This result is consistent with the finding that Dsh Δ DIX can mediate the noncanonical Wnt signaling in *Xenopus* and zebrafish (Heisenberg et al., 2000; Tada and Smith, 2000). The actin depolymerizing reagent, Latrunculin A, dispersed both RMA and Dsh Δ DIX to the cytosol. Essentially, the same result was also obtained using cytochalasin B (unpublished data). These results strongly suggest that Dsh interacts with F-actin either directly or indirectly and mediates the Wnt signaling to the actin cytoskeleton.

Noncanonical Wnt signaling pathway promotes protrusion formation and MARCKS is required for this activity

To examine whether the Wnt pathway regulates the protrusive activity, I coexpressed *Wnt11* and *Fz7 (Xenopus frizzled7)* in animal cap explants with the membrane-binding RFP. As shown in Fig. 2-13 A, the coexpression of Wnt11 and Fz7 dramatically promoted lamellipodia- and filopodia- like protrusions and it was inhibited by Xdd1, a dominant negative Dsh mutant (Sokol, 1996; Wallingford et al., 2000). When *MARCKS* MO was coinjected, this activity was severely inhibited (Fig. 2-13 A). It was rescued by coinjection with *MARCKS* mRNA. In addition, dorsal mesodermal cells expressing dominant-negative Wnt11 (Tada and Smith, 2000) significantly reduced the number of protrusions (Fig. 2-13 B), which is consistent with the observation by Wallingford et al. (2000) that cells expressing Xdd1 maintain significantly fewer stable protrusions. These results strongly suggest that the Wnt signaling pathway regulates cortical actin dynamics and that MARCKS is required for this process.

MARCKS is also required for the neural tube closure

When *MARCKS* MO was injected into the dorso-anterior blastomeres of eight-cell embryos to target the neuroectoderm, neural tube

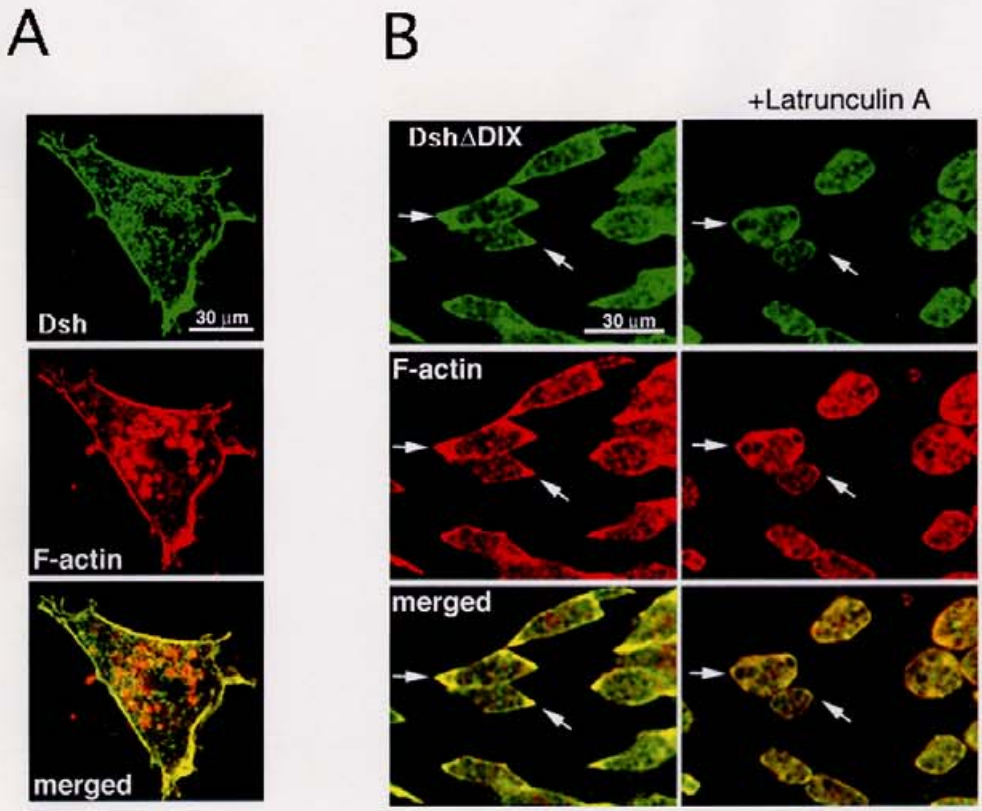

Figure 2-12. Dsh colocalized with cortical actin in the dorsal marginal zone cells.

(A) 250 pg of *Dsh-Venus* mRNA was expressed in DMZ explants. Cells were dissociated and plated on an FN-coated dish. Dsh-Venus was colocalized with the cortical actin. (B) 100 pg of *Dsh DIX-Venus* mRNA was expressed in DMZ explants. The explants were cultured on an FN-coated dish. F-actin was probed with RMA. Dsh DIX and RMA were colocalized (arrows). (Right) Treatment with 30 μ M Latrunculin A for 30 min.

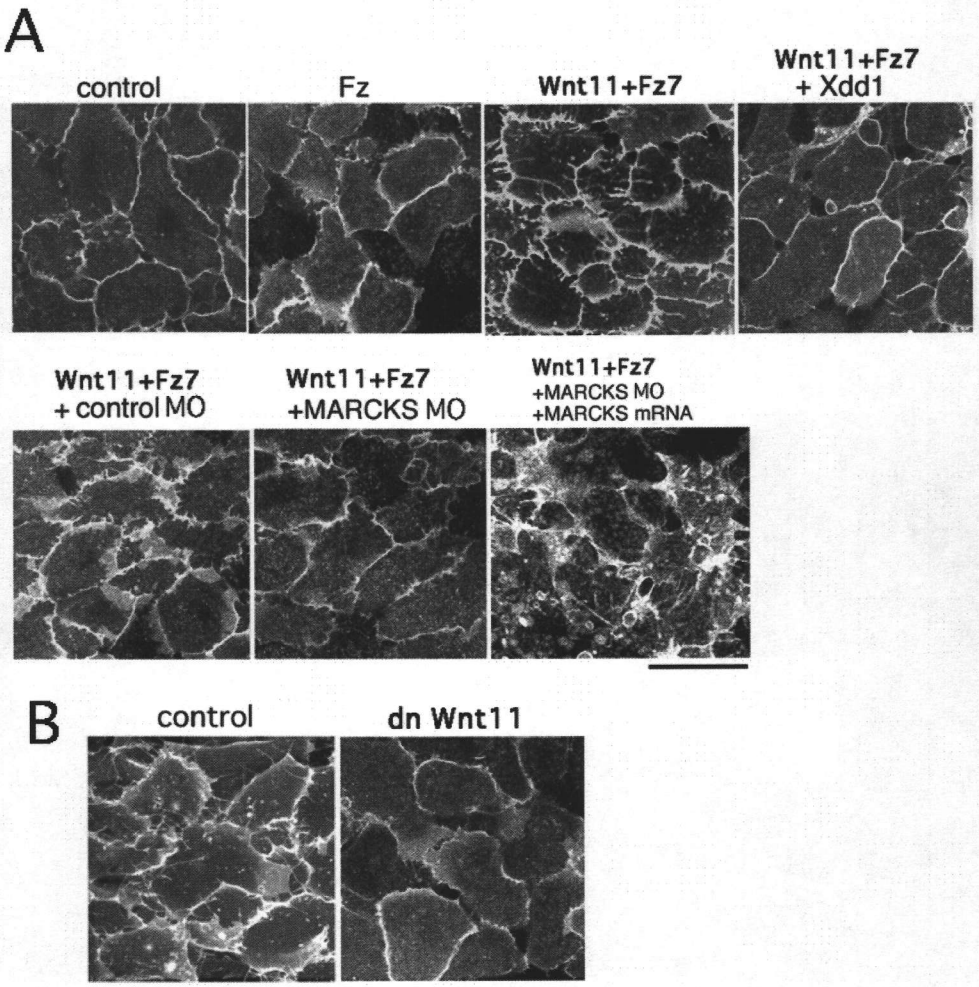

Figure 2-13. Noncanonical Wnt signaling pathway promotes protrusion formation and MARCKS is required for this activity.

(A) *Wnt11* and *Fz7* mRNAs (200 pg each) were coexpressed in animal cap explants with mb-RFP. The coexpression of *Wnt11* and *Fz7* promoted the protrusive activity. 5 pmol of *MARCKS* MO and Xdd1 inhibited it. The effect of *MARCKS* MO was rescued by coinjection of 200 pg of *MARCKS* mRNA. Bar, 50 μ m. (B) mb-RFP was injected with or without mRNA encoding dominant-negative Wnt-11 (2 ng). Bar, 50 μ m.

closure was severely impaired. This phenotype was rescued by coinjection of *MARCKS* mRNA, although the rescue was imperfect. This could be because the overexpression of *MARCKS* also impaired the neural tube closure. The pan-neural markers *XSox2* (Mizuseki et al., 1998) and *N-CAM* (Kintner et al., 1987). were expressed in the embryos injected with *MARCKS* MO as well as in the control embryos, suggesting that neural induction might not be affected. This result indicates that MARCKS is required for neural tube formation. It is consistent with the phenotype of mice deficient in MARCKS (Stumpo et al., 1995) and its close relative F52/MacMARCKS (Wu et al., 1996). MARCKS function may be conserved between mice and frogs.

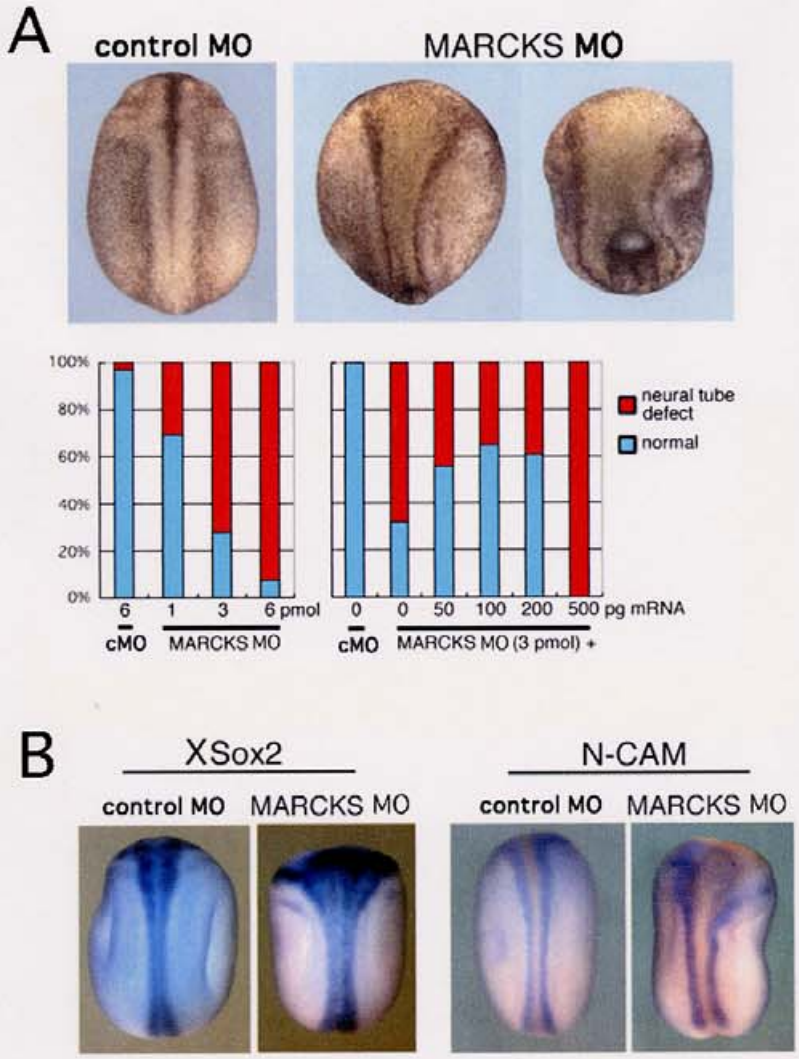

Figure 2-14. *MARCKS* MO inhibits neural tube closure.

(A) *MARCKS* MO was injected into the dorso-anterior blastomeres of eight-cell embryos to target the neuroectoderm, neural tube closure was severely impaired. This phenotype was rescued by co-injection of *MARCKS* mRNA, although the rescue was imperfect. (B) The pan-neural markers *XSox2* and *N-CAM* were expressed in the embryos injected with *MARCKS* MO as well as in the control embryos, indicating that neural induction was not affected.

DISCCUSION

Here, I have shown that MARCKS plays an essential role in regulating cortical actin dynamics in *Xenopus* development. *MARCKS* MO inhibited cell movements, cell shape change, cell adhesion, and interaction with FN, probably because of defect in the cortical actin dynamics. MARCKS is required not only for gastrulation but also for the neural tube formation. When *MARCKS* MO was injected into the dorso-anterior blastomeres of 8-cell embryos to target the neuroectoderm, neural tube closure was severely impaired (Fig. 2-14). It has been shown that *MARCKS* -deficient mouse shows neural tube closure defect (Stumpo et al., 1995), suggesting the conserved function between frogs and mice. It has been reported that *MARCKS-like protein* (*XMLP*) is also expressed in *Xenopus* embryo (Zhao et al., 2001). Although XMLP is similar to MARCKS (23% amino acid identity), *XMLP* -MO injected embryos showed malformations of the anterior axis and eye defect, but the gastrulation defect was not reported (Zhao et al., 2001). Thus, they seem to play distinct roles in *Xenopus* development.

The actin-binding domain of mammalian MARCKS binds to actin filaments and cross-links them *in vitro* (Hartwig et al., 1992). The corresponding domain of *Xenopus* MARCKS is 100% identical (Shi et al., 1997), suggesting that it may also interact with F-actin. MARCKS may be required for highly organized actin dynamics to effect dynamic tissue reorganization. The regulation of the cortical actin cytoskeleton by MARCKS may be important for a proper cellular response to signals such as Wnt and the FN/Integrin pathways. It is also possible that these signaling pathways regulate the activity of MARCKS. It has been shown that MARCKS is a PKC substrate. PKC has been involved in the noncanonical Wnt pathway (Sheldahl et al., 1999) and the Integrin pathway (Vuori and Ruoslahti, 1993). It would be interesting to determine how the activity of MARCKS is regulated during development.

CHAPTER 3

"Signaling mechanisms from Frizzled to Dishevelled in the PCP pathway that regulates convergent extension"

SUMMARY

One of the PCP signaling components Dsh plays a crucial role during gastrulation cell movements. It has been shown that Dsh is translocated to the plasma membrane in response to Fz7 signaling in the cells of animal cap explants. However, the molecular mechanism to signal from Fz7 to Dsh has not yet been elucidated. I identified molecules essential for this signal transduction. One of these molecules is PKC δ. Although PKC has been implicated in the Wnt signaling pathway, its molecular role is poorly understood. I identified novel genes encoding PKC δ in the *Xenopus* EST database. Loss of PKC δ function revealed that it is essential for convergent extension during gastrulation. Others are heterotrimeric G protein α subunits G_{i1} and G_{11}. Loss of function of these molecules inhibited both Dsh translocation in response to Wnt11/Fz7 signaling in animal cap cells and convergent extension movements in embryos. These G proteins and PKC δ are, thus, essential for signaling from Fz to Dsh in the PCP pathway.

INTRODUCTION

Three intracellular signaling pathways of the Wnts have been identified. These are Wnt/β-catenin pathway, PCP pathway and Wnt/Ca^{2+} pathway. Wnt/β-catenin pathway signals through Dishevelled and β-catenin. In *Xenopus*, the ectopic activation of this pathway induces a secondary axis. The PCP pathway, which regulates convergent extension, involves Dsh, Rho GTPases such as RhoA and Rac, and JNK. Wnt/Ca^{2+} pathway involved in the regulation of tissue separation during *Xenopus* gastrulation induces Ca^{2+} signaling through a heterotrimeric G protein.

The Wnt signaling pathway is mediated by a seven-transmembrane Wnt receptor, Frizzled, and the signal is transmitted through a cytoplasmic protein, Dsh which plays pivotal roles in the Wnt pathways (Boutros and Mlodzik 1999; Wharton 2003). In *Drosophila*, Dsh localizes to the membrane, and this localization is required for Dsh function (Axelrod 2001) in the PCP pathway. *Xenopus* Dsh is also translocated from the cytoplasm to the plasma membrane in response to a signal generated by some Frizzled receptors such as *Xenopus* Frizzled7 (Fz7) (Yang-Snyder et al. 1996; Axelrod et al. 1998; Rothbacher et al. 2000).

As discussed in Chapter 2, the PCP pathway regulates cortical actin dynamics. Thus, some of the PCP signal components must be localized and function in the cell cortex. Dishevelled is one of the candidate molecules,that it may recruit proteins to regulate cortical actin dynamics. It seems that the translocation of Dsh is specifically important for the PCP pathway because the membrane translocation of Dsh is not required for the activation of the canonical Wnt pathway (Rothbacher et al. 2000). However, the mechanism of this translocation and the activation of Dsh are unknown. Here, I raised a hypothesis that heterotrimeric G proteins and Protein kinase C (PKC) might be involved in this signal transduction.

PKC is thought to be involved in the Wnt signaling pathways for several reasons. Wnt5a and rat Frizzled2 activate the phosphatidylinositol pathway and increase the intracellular Ca^{2+} levels in zebrafish embryos (Slusarski et al. 1997a,b). The phosphatidylinositol pathway and Ca^{2+} levels

are closely related to PKC activation. In fact, overexpression of *Fz7* causes the translocation of epitope-tagged PKC from the cytoplasm to the plasma membrane in *Xenopus* embryos (Sheldahl et al. 1999; Medina et al. 2000). Kuhl et al. (2001) have shown that PKC phosphorylates Dsh *in vitro*. In addition, the loss of *Fz7* function leads to a defect in tissue separation during *Xenopus* gastrulation, which is rescued by the overexpression of PKC (Winklbauer et al. 2001). PKC is also implicated in the Wnt11 signaling pathway for *Xenopus* cardiogenesis (Pandur et al. 2002) and in the Dwnt4 pathway for *Drosophila* ovarian morphogenesis (Cohen et al. 2002). Although several lines of evidence suggest that PKC is involved in the Wnt signaling pathway, its molecular roles are poorly understood. The PKC family is subdivided into three subfamilies: the classical, novel, and atypical PKCs (cPKC, nPKC, and aPKC, respectively). cPKC is activated by Ca^{2+} and diacylglycerol (DAG), nPKC is activated by DAG but not by Ca^{2+}, and aPKC is not activated by these molecules (Kikkawa et al. 1989; Bell and Burns 1991; Nishizuka 1995; Newton 1997). I have demonstrated that PKC δ that belongs to the nPKC subfamily is essential for convergent extension, and that it regulates the function of Dsh in the PCP pathway.

Many seven-transmembrane receptors couple with heterotrimeric G proteins. Heterotrimeric G proteins consist of three subunits, α, β and γ. α subunits bind to a guanine nucleotide. A GDP-bound form of α subunit forms a complex with β and γ subunits in an inactive state. Upon activation, an α subunit exchanges the guanine nucleotide from GDP to GTP, dissociates from β/γ subunits, and activates downstream signaling. Because the Frizzled is a seven-transmembrane receptor, it is very likely that it may signal through G proteins. Malbon and his colleagues used chimeric receptors which have the extracellular domains of β-adrenergic receptor fused to the intracellular domains of Frizzled (Liu 1999,2001). In this system, they have shown that the Wnt/β-catenin pathway requires $G\alpha_q$ and $G\alpha_o$, and that the Wnt/Ca^{2+} pathway requires $G\alpha_o$ and $G\alpha_t$. In *Xenopus*, it is unknown whether G proteins are involved in the Wnt pathways. Thus, I examined loss of function experiments using MOs against

some Gα subunits expressed in *Xenopus* embryos and found Gα_{11} and Gα_{i1} are essential for the PCP pathway.

EXPERIMENTAL PROCEDURES

Plasmids, RNA synthesis, and morpholino oligos

Our *Xenopus* EST database (NIBB XDB, http://Xenopus.nibb. ac.jp) was searched with the cDNA sequences of mammalian nPKC family members using BLAST. Full-length cDNA clones, XL011f02 and XL066d07, were identified. These clones were sequenced and designated PKCδ1 and PKCδ2, respectively. GenBank accession numbers are AB109739 and AB109740, respectively. Plasmids for the expression in *Xenopus* embryos were constructed with PCR products inserted into the expression vector pCS2+. Capped mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion). PKCδ ΔC contained the N-terminal regulatory domain (1–347 amino acids) of PKCδ1. A plasmid bearing the gene for constitutively active MKK7 (MKK7 DED) was a kind gift from Dr. E. Nishida (Kyoto University, Japan). The plasmid bearing the gene for GAL4(DBD)-tagged c-Jun was a kind gift from Dr. M. Tada (National Institute for Medical Research, UK). The myc-Dsh was a kind gift from Dr. R. Harland (University of California, Berkeley). For the Venus-tagged constructs, the indicated fragments were amplified with PCR, fused to Venus gene (Nagai et al. 2002), and sequenced. For mb-Venus, a cDNA fragment of the C-terminal region (158–188 amino acids) of *Xenopus* K-ras (Baum and Bebernitz 1990) was cloned by PCR using the plasmid XL213p09 (NIBB XDB) as a template. Dsh DEP contained the N-terminal region (1–426 amino acids). *Xenopus* Rac (GenBank accession no. AF174644) was cloned by PCR using neurula cDNA. Arp3 was cloned by PCR using the plasmid XL019o05 (NIBB XDB). PKCδ and Arp3 were fused to the N terminus of Venus, and Dsh and Dsh DEP were fused to the C terminus. Antisense morpholinos were obtained from Gene Tools. The morpholino oligo sequences were as follows: PKCδ1 MO, 5 –AGGATATGCGTAGGAAGGAGACATG-3 ; PKCδ2 MO, 5-AGGATAAGCGTAGGAAAGGAGCCAT-3 ; Control MO, 5 –CCTCTT ACCTCAGTTACAATTTATA-3 .

In situ hybridization and RT–PCR analysis

In situ hybridization in *Xenopus* was carried out as described in Harland (1991). The detection of β-galactosidase activity for tracing cell lineage was carried out as described by Kurata and Ueno (2003). For RT–PCR analyses, RNA from *Xenopus* embryos was prepared with Trizol (Life Technologies). cDNA was synthesized with Reverse Transcriptase (#TRT-101, Toyobo). Sequences of the primers for *Xbra*, *Xnr3*, and *Xotx2* were as described in Yamamoto et al. (2001) and those for *chordin*, *goosecoid*, *siamois*, and *ODC* were as described in Dr. De Robertis' home page (http://www.hhmi.ucla.edu/derobertis/index.html). Primers for *Xmyf5* were 5 -CAGAATGGAGATGG TAGATAGC-3 and 5 –AGCCTGGTTCACTTTCTT TAGC-3 ; those for *Wnt11* were 5 -AAGT-GCCACGGAGTGTCT GG-3 and 5 -CTCAGACTCTCTCACTGGCC-3 ; and those for *PKC* were 5 –TTTATTA ACCCCAAGATGGAGCG-3 and 5 -AACTACATTCAAGTAACCAG-3 .

Whole-mount immunostaining and immunocytochemistry of *Xenopus* embryos

The procedure for whole-mount immunostaining was as described in Kurata et al. (2001). The antibodies were MZ15 for notochord (a kind gift from Dr. F. Watt; Smith and Watt 1985) and 12/101 for somites (Development Studies Hybridoma Bank; Kintner and Brockes 1984). As secondary antibodies, horseradish peroxidase-conjugated and alkaline phosphatase-conjugated antibodies were used for MZ15 and 12/101, respectively. For immunocytochemistry, each epitope-tagged mRNA was injected into the animal pole of two-cell embryos. The animal caps were dissected from stage 9–10 embryos and fixed with MEMFA, followed by immunostaining by a standard method using a fluorescence-labeled secondary antibody. The localizations were determined by laser-scanning confocal microscopy, using a Carl Zeiss LSM510 microscope. The antibodies for immunocytochemistry were anti-myc 9E10 (Boehringer Mannheim) and rabbit polyclonal anti-flag (Sigma) antibodies. For PMA treatment, phorbol 12–myristate 13–acetate (#P1585, Sigma) was used.

Elongation assay in *Xenopus* animal cap and DMZ explants

For the animal cap explants, mRNAs or a morpholino oligonucleotide were coinjected with 0.5 pg activin mRNA into the animal pole of two-cell embryos. The animal cap was dissected manually from stage 9 embryos. For DMZ explants, mRNA or a morpholino oligonucleotide were injected into the two dorsal blastomeres of four-cell embryos. Explants were isolated at stage 10+. These explants were cultured in 0.1% BSA/1× Steinberg' s solution until sibling embryos reached stage 17. The procedure for observing cells during convergent extension movements was basically according to Wallingford et al. (2000) with some modifications. Explants were isolated at stage 10+ and cultured in 1× Steinberg' s solution on a cover glass coated with fibronectin. The explants were observed by laser-scanning confocal microscopy.

Immunoprecipitation and Western blotting

HEK293T cells were transiently transfected with the indicated constructs using Lipofectamine Plus (Invitrogen). Cell lysates were prepared in PBS containing 0.1% Triton-X100, 20 mM NaF, 0.5 mM PMSF, and a 1/200 volume of protease inhibitor cocktail (#P8340, Sigma), and spun at 15,000*g* for 10 min. The indicated antibodies were added to the supernatants, and incubated at 4° C overnight. Protein A/G agarose (#SC-2003, Santa Cruz Biotechnology) was added, and the mixture was incubated for 1 h in a tumbling mixer. The agarose beads were washed five times with the lysis buffer. The antibodies used for immunoprecipitation and Western blotting were anti-myc 9E10 (Boehringer Mannheim), anti-flag monoclonal M2 (Sigma), and anti-GFP (#598, Molecular Biological Laboratories) antibodies.

JNK assay

mRNA encoding GAL4 (DBD)-tagged c-Jun (100 pg) was injected into two-cell embryos. The animal caps were isolated at stage 10 and smashed by pipetting in sample buffer for SDS-PAGE. These samples were boiled and fractionated by SDS-PAGE. Western blotting was performed

using anti-GAL4 (DBD; #SC-510, Santa Cruz Biotechnology) and anti-phospho-c-Jun (#9261S, Cell Signaling) antibodies.

RESULTS

PKC δ is expressed during *Xenopus* embryogenesis

Although PKC δ has been implicated in the noncanonical Wnt signaling pathway, its molecular role is poorly understood. It has been shown that Wnt5a and rat Frizzled2 triggers the phosphatidylinositol pathway and induce an increase in intracellular Ca^{2+} (Slusarski et al. 1997a,b). Among the PKC subfamilies, cPKC and nPKC are known to be activated by Ca^{2+} and/or diacylglycerol (DAG). For this reason, I searched our *Xenopus* EST database (NIBB XDB, http://Xenopus.nibb.ac.jp) to identify PKC family members that belong to the cPKC or nPKC subfamily. I found that in addition to *PKCα* and *PKCβ*, which have already been reported (Chen et al. 1988), the database included two novel cDNAs encoding nPKC family members. The predicted amino acid sequences of these two PKCs had 95% identity, suggesting that these are duplicated genes due to the tetraploidism of *Xenopus laevis*. As described later, these two genes had indistinguishable activities in the tests I performed. These proteins are the most similar to mammalian δ-type PKC. Thus, I designated these genes *PKCδ1* and *PKCδ2*. It is known that the N-terminal regulatory domain of PKCs inhibits the kinase activity by masking the catalytic domain, and activators such as DAG release this autoinhibition by binding to the C1 domain (Kemp et al. 1994; Orr and Newton 1994; Nishizuka 1995; Newton 1997). The regulatory domain of *Xenopus* PKC δ 1/2, including the C1 domain, is highly homologous to that of human PKC δ, suggesting that these regulatory mechanisms are conserved. PKC δ is relatively similar to PKC θ and PKC ε, which also belong to the nPKC family. This class of PKCs is found not only in vertebrates, but also in sea sponges (GenBank accession no. CAA73557), *Aplysia* (GenBank accession no. 16975), *Hydra* (GenBank accession no. CAA72926), *Drosophila* (GenBank accession no. NP_511171), and nematodes (GenBank accession no. NP_499860). Thus, the nPKCs may have evolutionally conserved regulatory mechanisms and functions distinct from those of other PKC subfamilies. To determine the expression patterns

during *Xenopus* development, I performed reverse transcriptase PCR (RT–PCR) using primers whose sequences were common to *PKCδ1* and *PKCδ2*. As shown in Figure 3-1 A, *PKCδ* was expressed from the two-cell stage through the tadpole stage. In situ hybridization using probes for *PKCδ1* and *PKCδ2* revealed that they were ubiquitously expressed (Fig. 3-1 B). *PKC δ 1* and *PKC δ 2* were strongly expressed in the mesoderm and ectoderm during gastrulation, indicating their possible involvement in the regulation of gastrulation movements.

Overexpression of PKCδ lacking the catalytic domain inhibits gastrulation movements

To test whether PKCδ is involved in the regulation of gastrulation movements, I made an expression construct for PKCδ1 lacking the catalytic domain (PKCδΔC). The N-terminal regulatory domain of PKCs includes pseudosubstrate and C1 domains. The pseudosubstrate domain interacts with the kinase domain and inhibits the catalytic activity (Kemp et al. 1994; Orr and Newton 1994). The C1 domain interacts with DAG and other activators. A mutant lacking the catalytic domain was expected to function as a dominant-negative form by binding to the catalytic domain of a native protein through its pseudosubstrate domain and/or by competitive binding to the activators. RNA encoding PKCδΔC was synthesized in vitro and injected into the two dorsal blastomeres of four-cell embryos. As shown in Figure 3-2, PKC δ Δ C severely inhibited gastrulation movements. Involution of the mesoderm was impaired, and the blastopore remained open or showed delayed closing. The same phenotype was observed in embryos injected with *PKCδ2* lacking the catalytic domain (data not shown). The phenotype was rescued by full-length *PKCδ1* (Fig. 3-2) or full-length *PKCδ2* (data not shown), suggesting that *PKC δ Δ C* functioned as a dominant-negative mutant of *PKCδ*. It has been reported that a similar gastrulation-defect phenotype is caused by loss-of-function of the noncanonical Wnt signaling components, such as Wnt11 (Tada and Smith 2000) and Fz7 (Djiane et al. 2000). To test whether PKCα or PKCβ has

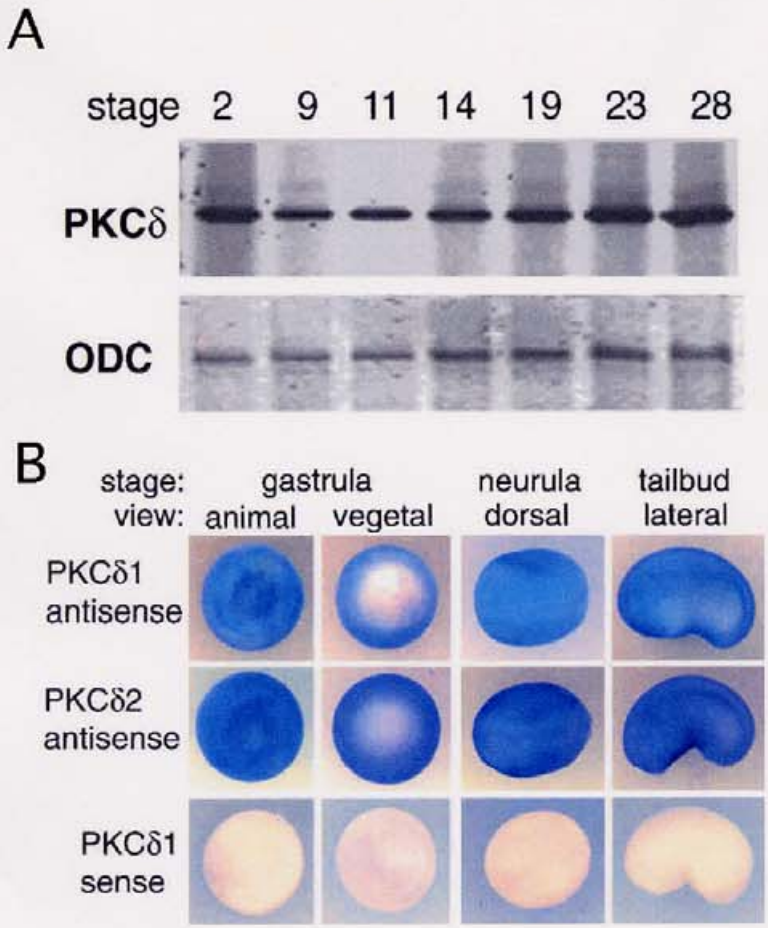

Figure 3-1. PKC δ is expressed during *Xenopus* embryogenesis.

(A) RT-PCR analysis of *PKC δ* expression during *Xenopus* development. Primers whose sequences were common between *PKC 1* and *PKC 2* were used. Stages are according to Nieukoop and Faber (1994). (B) In situ hybridization probing with *PKC δ 1* and *PKC δ 2* showing their ubiquitous expression.

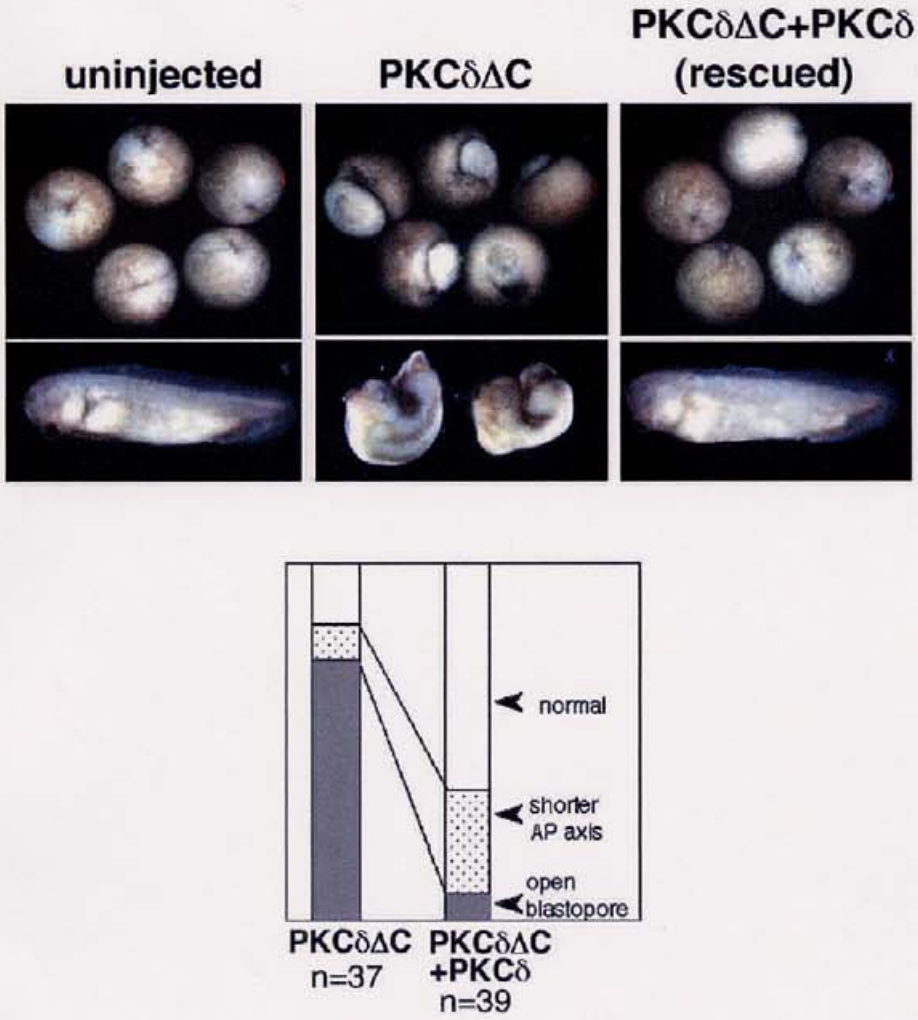

Figure 3-2. Overexpression of PKC δ lacking the catalytic domain inhibits gastrulation movements.

PKC δ 1 lacking the catalytic domain (PKC δ ΔC) inhibits gastrulation movements. RNA (100 pg) encoding PKC δ ΔC was injected into the two dorsal blastomeres of four-cell embryos. PKC δ ΔC severely inhibited gastrulation movements, and this effect was rescued by the coinjection of 1 ng of RNA encoding full-length PKC δ 1. Embryos in the top panels are at the early neurula stage.

an activity similar to that of PKC δ, I constructed mutant genes encoding PKC α or PKC α lacking the catalytic domain (PKC α ΔC and PKC β ΔC, respectively), expecting that they would function as dominant-negative mutants of their respective native forms. I injected the same amount of mRNA encoding PKC α ΔC, PKC β ΔC, or PKC δ ΔC into *Xenopus* embryos. Although comparable levels of the mutant proteins were detected by Western blotting, PKC α ΔC and PKC β ΔC did not have any effects on gastrulation, unlike PKC δ ΔC (data not shown). This result suggests that the role in gastrulation movements may be PKC δ-specific. To determine whether this gastrulation-defective phenotype was caused by a defect in mesodermal differentiation, the expression of mesodermal markers, I used in situ hybridization to examine a pan-mesodermal marker, *Xbra*, and dorsal mesodermal markers *chordin* (chd) and *goosecoid* (gsc). At the gastrula stage, *PKC δ Δ C*-injected embryos expressed these markers at the same level as control embryos (Fig. 3-3 A). In tadpoles, the notochord and somites were differentiated in the *PKC δ Δ C*-injected embryos, but the extension of these tissues was severely inhibited (Fig. 3-3 B,C). These results indicated that the phenotype was caused not by a defect in mesoderm differentiation, but by a defect in morphogenetic movements. I then tested whether *PKC δ Δ C* inhibited the elongation of animal caps by activin. In this system, when the dorsal mesoderm is induced in animal cap explants by activin, the explants elongate by convergent extension movements. *PKC δ Δ C* expression inhibited the elongation of explants by activin without affecting the induction of mesodermal markers (Fig. 3-4 A,B). A similar effect has been observed as a result of inhibition of the noncanonical Wnt pathway. Our results suggest that PKC δ is also required for the convergent extension.

PKC δ antisense morpholino also blocked gastrulation movements and convergent extension

To confirm that the effects of *PKC δ Δ C* were due to the depletion of PKC δ activity, I made antisense morpholino oligonucleotides (MOs) for *PKC*

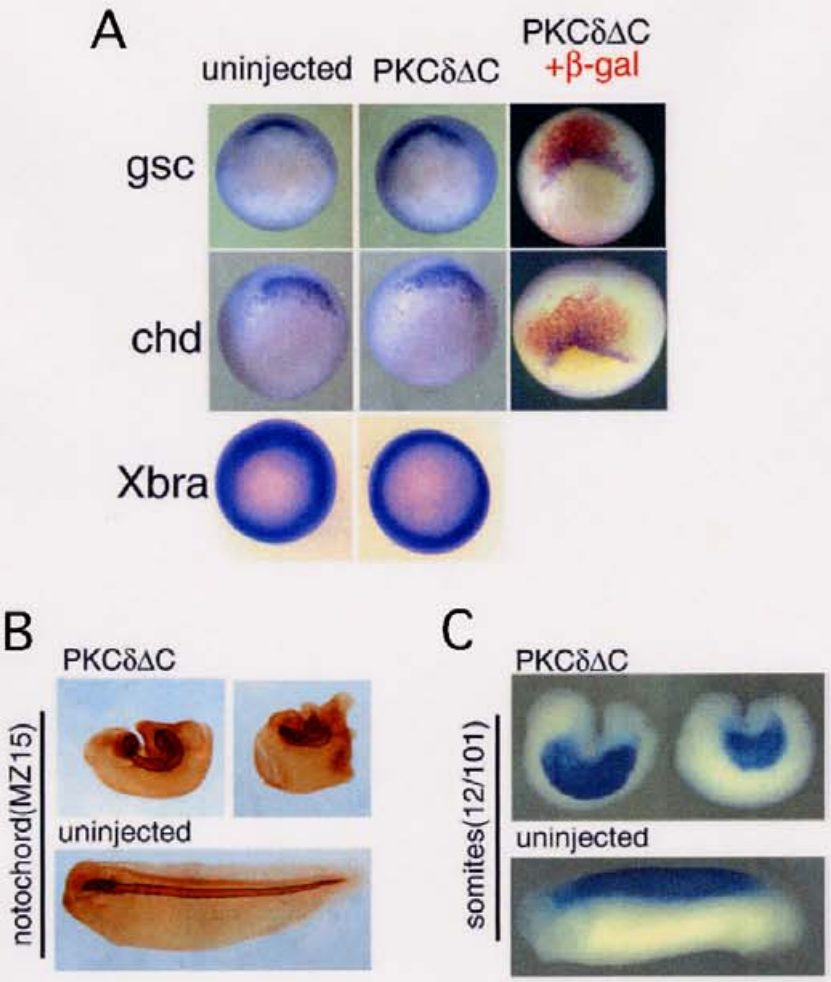

Figure 3-3. Mesodermal induction was not affected by *PKC δ Δ C*.

(A) In situ hybridization of early gastrula embryos probed with a pan-mesodermal marker, *Xbra*, and dorsal mesodermal markers *chordin* (chd) and *goosecoid* (gsc). (*Left*) Uninjected. (*Middle*) *PKC δ Δ C* RNA (200 pg) was injected into all four blastomeres of four-cell embryos. (*Right*) In order to trace the cell lineage, mRNA encoding β-galactosidase (β-gal) with a nuclear localization signal was coinjected with *PKC δ Δ C* into two dorsal blastomeres of four-cell embryos. Cells expressing *β-gal* were stained in red. (B,C) Immunostaining of the notochord and somites in *PKC δ Δ C*-injected embryos.

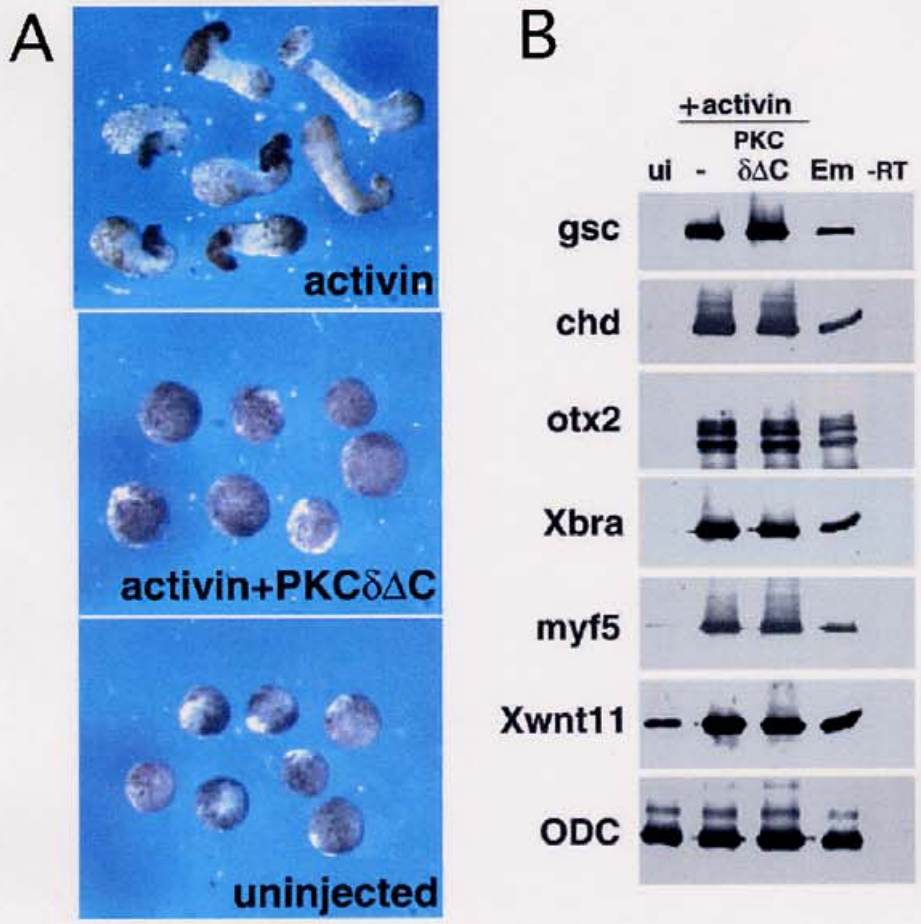

Figure 3-4. ***PKC δ Δ C*** **affect the mesodermal elongation but not induction.**
(A) The *PKC δ Δ C* mutant blocked the elongation of animal cap explants by activin. Activin RNA (0.5 pg) was injected with or without 100 pg of *PKC δ Δ C* RNA. (B) The induction of mesodermal markers by activin in animal caps was not affected by PKC δ Δ C. ui, uninjected; Em, whole embryo; gsc, *goosecoid*; chd, *chordin*.

δ and tested their effects on development. Because the highly homologous *PKCδ1* and *PKCδ2* are both expressed, MOs for both *PKCδ1* and *PKCδ2* were prepared. First, I confirmed that these MOs inhibited the translation of mRNA that has the corresponding sequences. As shown in Figure 3A, each MO blocked the production of its respective GFP-tagged PKC δ but unrelated GFP was not affected. To inhibit PKC δ synthesis in embryos, the MOs for *PKCδ1* and *PKCδ2* were mixed at an equimolar ratio and injected into four-cell embryos (the mixed Mos will be referred to as *PKCδ*MO hereafter). The *PKCδ*MO caused a gastrulation-defective phenotype that was indistinguishable from that of *PKCδΔC* -injected embryos (Fig. 3-5). This phenotype was efficiently rescued by flag-tagged *PKCδ1*, which do not have 5 -UTR binding to the MOs. In addition, MO-injected embryos expressed the mesodermal markers *Xbra*, *chordin*, and *goosecoid*, suggesting that the mesoderm differentiation was not affected (Fig. 3-6 A). The similarity of the phenotypes of the embryos injected with *PKCδΔC* and *PKCδ* MO indicated that PKC δ ΔC functioned as a dominant-negative mutant for PKC δ. To investigate the role of PKC δ in convergent extension, I examined the effect of *PKCδ* MOs on the elongation of dorsal marginal zone (DMZ) explants. As shown in Figure 3-6 B, the *PKCδ* MO inhibited the elongation of these explants, supporting the idea that PKC δ may be required for convergent extension.

PKC δ is translocated to the membrane in response to Fz7 signaling and interacts with Dsh

The inhibition of convergent extension by the loss of PKC δ function strongly implies that PKC δ plays a role closely related to the noncanonical signaling pathway. It has been shown that Fz7 translocates Dsh and PKC δ from the cytoplasm to the plasma membrane in *Xenopus* embryos (Axelrod et al. 1998; Sheldahl et al. 1999; Medina and Steinbeisser 2000; Medina et al. 2000). To examine whether Fz7 also regulates the subcellular localization of PKC δ, flag-tagged *PKCδ* and myc-tagged *Dsh* were coexpressed with or without *Fz7* in animal cap explants of *Xenopus* embryos. The localization of the tagged proteins was then observed with a

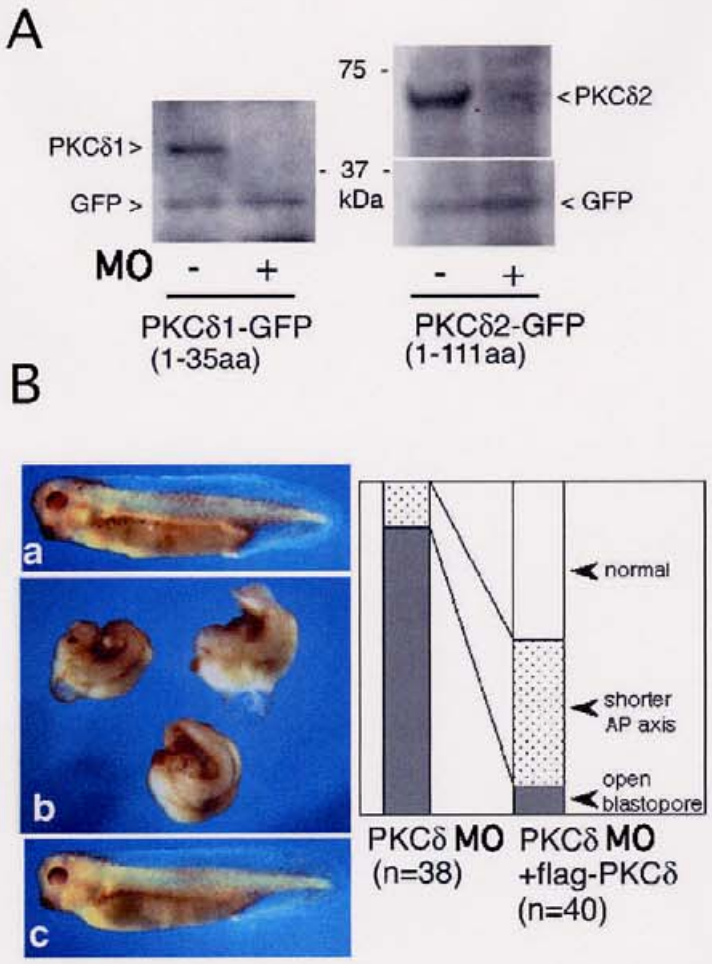

Figure 3-5. *PKC δ* MO induced gastrulation defect in early *Xenopus* embryo.
(*A*) Morpholino oligonucleotides (MOs) for *PKC δ 1* and *PKC δ 2* inhibited the translation of mRNA that had the corresponding sequences. RNA encoding GFP-tagged PKC δ and unrelated GFP were coinjected with or without each MO. *PKC δ 1* (*left* panel) and *PKC δ 2* MO (*right* panel) blocked the production of each GFP-tagged PKC δ , but unrelated GFP was not affected. (*B*) Control MO or *PKC δ* MO (20 ng each) was injected into four-cell embryos (panels *a*,*b*, respectively). The *PKC δ* MO caused a gastrulation-defective phenotype that was indistinguishable from that of *PKC δ* Δ *C*-injected embryos. This phenotype was rescued by 1 ng of full-length *PKC δ 1* RNA (panel *c*).

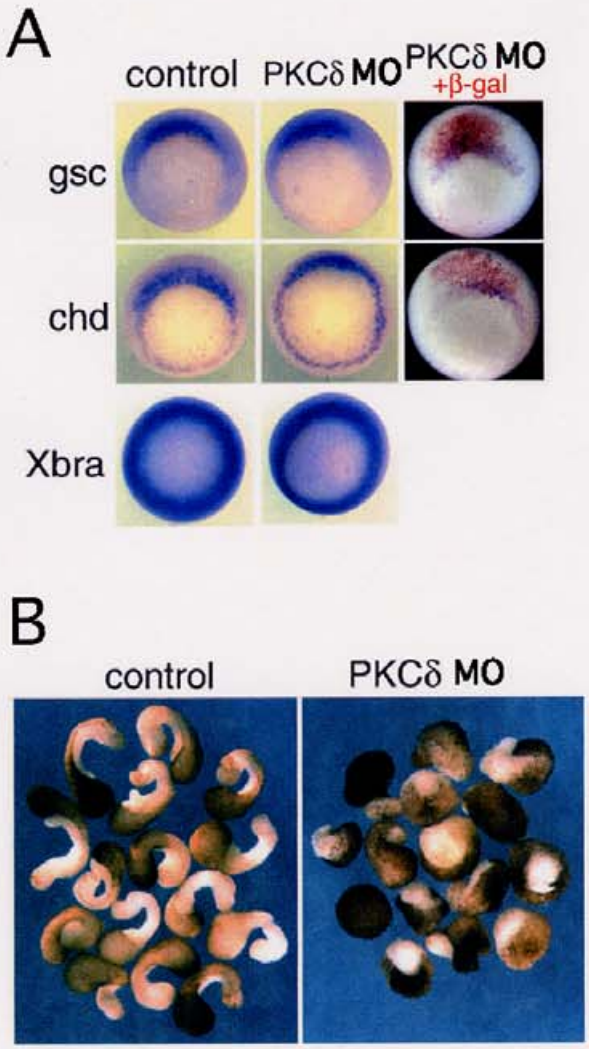

Figure 3-6. PKC δ antisense morpholino also blocked gastrulation movements. (A) *In situ* hybridization of early gastrula embryos probed with *chordin* (chd), *goosecoid* (gsc), and *Xbra*. The *left* and *middle* panels show 20 ng of control or *PKC δ* MO was injected into all four blastomeres of four-cell embryos. (*Right*) To trace the cell lineage, mRNA encoding -gal with a nuclear localization signal was coinjected with *PKC δ* MO into two dorsal blastomeres of four-cell embryos. (B) *PKC δ* MO inhibited the elongation of dorsal marginal zone explants. Twenty nanograms of MO were injected into the two dorsal blastomeres of four-cell embryos.

laser-scanning confocal microscope (Fig. 3-7). In the absence of *Fz7* mRNA, PKC δ and Dsh were mainly in the cytoplasm. Interestingly, however, when *Fz7* was coexpressed, they were mostly localized to the plasma membrane. In general, this class of PKC binds to and is activated by DAG that is produced on the membrane upon extracellular signaling. Thus, DAG may be produced by Fz7 and then localize PKC δ to the membrane, which further implies that PKC δ is involved in the Wnt/Fz7 pathway. The above finding that the cotranslocation of PKC δ and Dsh from the cytoplasm to the membrane depended on Fz7 function prompted us to examine whether these proteins might interact with each other. To test this possibility, flag-tagged *PKC δ* and myc-tagged *Dsh* were expressed in HEK293T cells and immunoprecipitation was performed. As shown in Figure 3-8 A, PKC δ and Dsh were coimmunoprecipitated. When the flag and myc tags on *PKC δ* and *Dsh* were exchanged, I obtained essentially the same result (data not shown). These findings indicated that PKC δ and Dsh form a complex. To test whether the activation of PKC δ by the phorbol ester PMA (phorbol 12–myristate 13–acetate) altered this binding property, I treated transfected HEK293T cells with PMA and performed an immunoprecipitation. PMA is known to be a potent activator for PKC δ and other members of the novel and classical PKC subfamilies (Kikkawa et al. 1989; Bell and Burns 1991; Zhang et al. 1995). As shown in Figure 3-8 B, PMA treatment did not change the amount of coimmunoprecipitated Dsh and PKC δ. In addition, a kinase-negative mutant of PKC δ was also coimmunoprecipitated with Dsh in HEK293T lysates (data not shown), indicating that this physical interaction may not depend on the activity of PKC δ. Taken together, these results suggest that PKC δ and Dsh form a complex, and this complex is translocated to the membrane upon activation of the Fz7 signal.

PKC δ is required for Dsh activation by Fz7 signaling

The precise molecular mechanisms of the membrane localization of Dsh by Wnt/Fz7 signaling are not known. To investigate this issue, I next tested whether PKC δ is required for the Fz7-dependent membrane

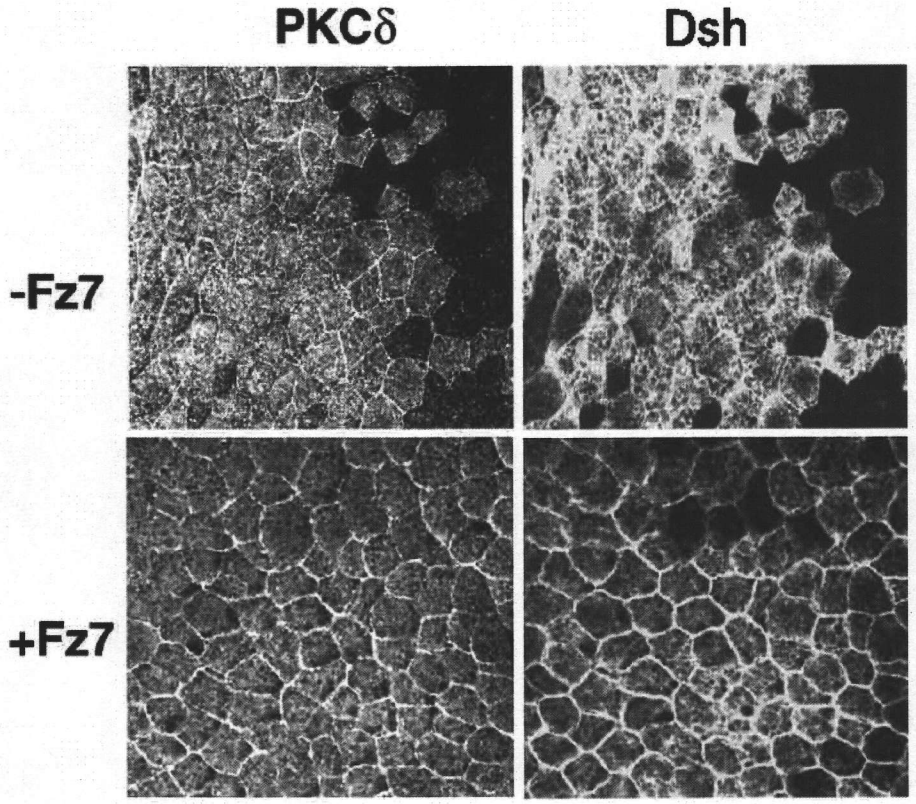

Figure 3-7. PKC δ is localized to the plasma membrane with Dsh by Fz7 signaling.

Flag-tagged *PKC δ* mRNA (200 pg) and myc-tagged *Dsh* mRNA (100 pg) were coinjected with or without 500 pg of *Fz7* mRNA in the animal cap explants of *Xenopus* embryos. Their localization was observed by laser-scanning confocal microscopy.

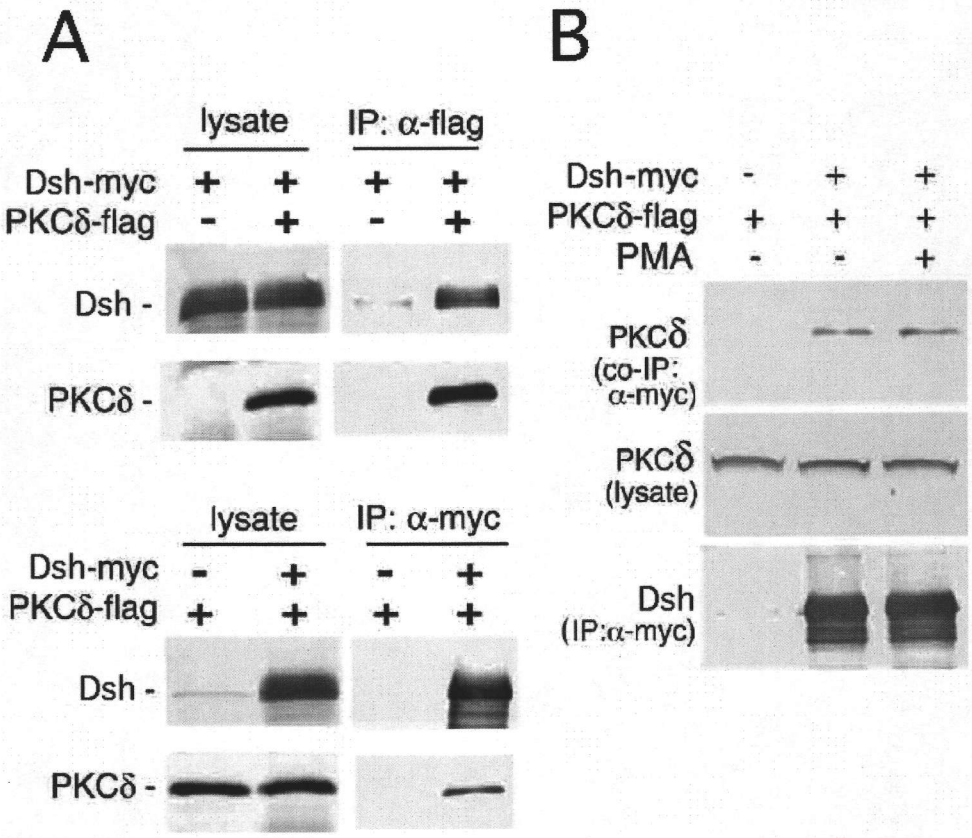

Figure 3-8. Dsh and PKC δ are physically interacted.

(A) Coimmunoprecipitation of PKC δ and Dsh. Flag-tagged *PKC δ* and myc-tagged *Dsh* were expressed as indicated in HEK293T cells. PKC δ and Dsh coimmunoprecipitated, indicating that they form a complex. (B) The indicated genes were expressed in HEK293T cells. PMA was added to the medium at a final concentration of 1 μM 2 h before the cell lysate preparation. The addition of PMA did not change the amount of coimmunoprecipitated Dsh and PKC δ.

localization of Dsh. *PKCδ* MO was coinjected with myc-tagged *Dsh* and *Fz7* mRNAs, and localization of Dsh to the animal cap cells was observed. As shown in Figure 3-9, the coinjection of *PKCδ* MO blocked the membrane localization of Dsh in response to Fz7. Dsh is a phosphoprotein whose phosphorylated state is elevated (hyperphosphorylated) upon the activation of the noncanonical Wnt signaling pathway (Yanagawa et al. 1995; Willert et al. 1997; Rothbacher et al. 2000; Tada and Smith 2000). This increase in phosphorylation can be monitored by a mobility shift of the Dsh protein in SDS polyacrylamide gel electrophoresis (SDS-PAGE). To test whether PKC δ affects the phosphorylation state of Dsh, myc-tagged *Dsh* mRNA was coinjected with *PKCδ* MO or *PKCδΔC* mRNA into four-cell embryos. Animal cap explants were isolated at around stage 10, and their extracts were subjected to SDS-PAGE (Fig. 3-10 A). The myc-tagged Dsh protein was detected by Western blotting using an anti-myc antibody. Two bands were detected in the *Dsh*-injected samples. In the absence of *Fz7*, the lower band was more intense than the upper band. When *Fz7* was coinjected, the upper band became much more intense. This indicated that Dsh was hyperphosphorylated by Fz7 signaling. The coinjection of *PKCδΔC* or *PKC δ* MO blocked this hyperphosphorylation of Dsh. These results indicated that PKC δ is required for both the membrane localization and the phosphorylation of Dsh, suggesting that PKC δ is essential for the signaling from Fz7 to Dsh. If the Dsh function requires PKC δ activity, the activation of JNK in the noncanonical Wnt pathway should be blocked by the loss of PKC δ function. To examine this possibility, I assayed the JNK activity. GAL4 DNA binding domain (DBD)-tagged c-Jun mRNA was injected into *Xenopus* embryos, and the phosphorylation level of c-Jun was assessed by Western blotting using anti-phosphorylated- c-Jun and anti-DBD antibodies. As shown in Figure 3-10 B, the overexpression of PKC δ or Fz7 alone slightly activated the JNK activity. However, the activity was greatly enhanced by the coexpression of *Fz7* and *PKCδ*. Moreover, *PKCδ* MO blocked the activation of JNK by Fz7. These results indicated that PKC δ is required for the activation of JNK by Fz7 signaling.

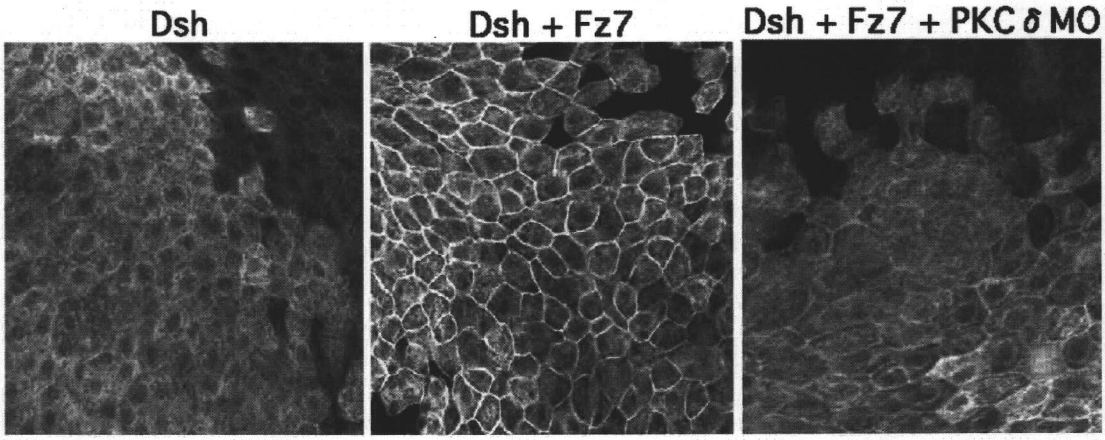

Figure 3-9. PKC δ is required for Dsh translocation in response to Fz7 signaling.

Twenty nanograms of *PKC δ* MO were coinjected with 100 pg of myc-tagged *Dsh* and 500 pg of *Fz7* mRNAs, and the localization of Dsh in animal cap explants was observed. The coinjection of *PKC δ* MO blocked the membrane localization of Dsh by Fz7.

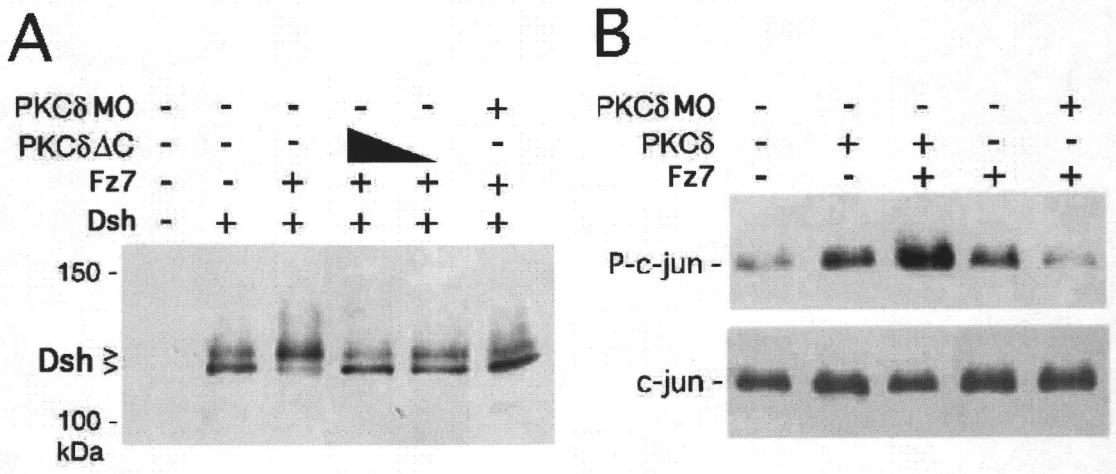

Figure 3-10. PKC δ is required for the activation of Dsh by Fz7 signaling.
(A) Myc-tagged *Dsh* and *Fz7* mRNAs were coinjected with *PKC δ* MO or *PKC δ Δ C* mRNA. Animal cap explants were isolated at stage 10, and their extracts were fractionated by SDS-PAGE. Myc-tagged Dsh protein was detected by Western blotting using an anti-myc antibody. Two bands were detected in all four lanes for *Dsh*-injected samples. (B) PKC δ is required for JNK activation by Fz7. GAL4 DNA-binding domain (DBD)-tagged *c-Jun* mRNA was injected, and the phosphorylation levels of c- Jun were detected by Western blotting using anti-phosphorylated c-Jun (P-c-Jun) and anti- DBD antibodies (c-Jun).

Activation of PKC δ is sufficient for Dsh translocation and for activation of the JNK pathway

As described above, PKC δ and Dsh form a complex, and both are translocated to the plasma membrane upon the activation of the noncanonical Wnt pathway. I postulated that PKC δ recruits Dsh to the membrane in this process. If this is true, the activation of PKC δ might be sufficient for the translocation of Dsh. To test this possibility, I injected RNAs encoding flag-tagged *PKC δ 1* and myc-tagged *Dsh* into *Xenopus* embryos, and the animal caps were explanted and treated with PMA. PMA is a functional analog of DAG that activates PKC on the membrane by binding to the C1 domain. As shown in Figure 3-11 A, both PKC δ and Dsh were translocated to the plasma membrane by PMA within 15 min. In addition, PMA treatment activated the JNK in the animal cap explants (Fig. 3-11 B). Thus, PKC δ activation is sufficient for Dsh translocation and the activation of downstream signaling.

The PKC δ loss-of-function phenotype is partially rescued by the overexpression of active MKK7

I have shown that PKC δ is required for the activation of JNK by Fz7. If the gastrulation-defective phenotype caused by *PKC δ* MO is due to the blockade of JNK activation, it might be rescued by the overexpression of *MKK7*, which is known to activate JNK directly. To examine this possibility, I coinjected *PKC δ* MO with constitutively active (CA) *MKK7* (Yamanaka et al. 2002). Closure of the blastopore of the injected embryos was compared at stage 14, which is when the blastopore in the control embryos was completely closed. *PKC δ* MO blocked the gastrulation movement in more than 90% of the injected embryos (Fig. 3-12 A). *PKC δ* and CA *MKK7* mRNAs rescued the phenotype completely and partially, respectively, suggesting that MKK7/JNK functions at least in part downstream of PKC δ. This result supported the idea that Fz7 regulates JNK activity through PKC δ. At the tadpole stage, the embryos rescued by *MKK7* showed short trunks and defects in head formation (Fig. 3-12 B). This partial rescue suggests that

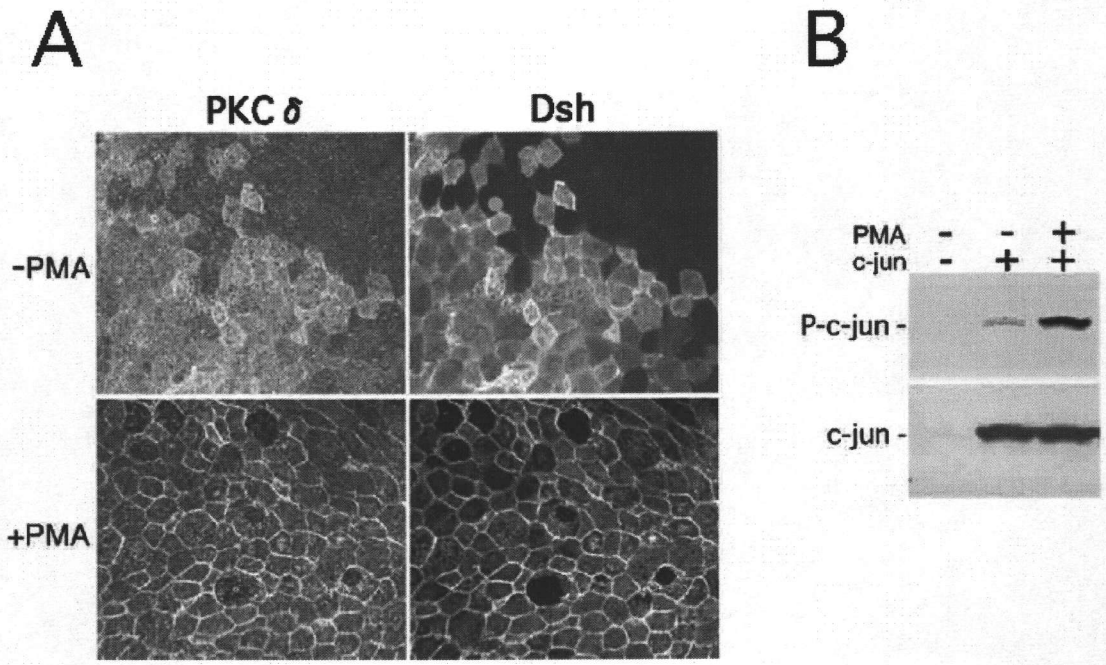

Figure 3-11. PKC δ activation is required for Dsh translocation.

(A) Animal cap explants expressing myc-tagged *Dsh* and flag-tagged *PKC δ* were treated with PMA, and the localization of Dsh and PKC δ was observed. Dsh was translocated to the plasma membrane by PMA. (B) PMA can activate JNK in animal cap explants. Isolated explants were treated with or without 1 μM PMA for 1 h. The JNK activity was detected by an anti-phospho- c-Jun antibody.

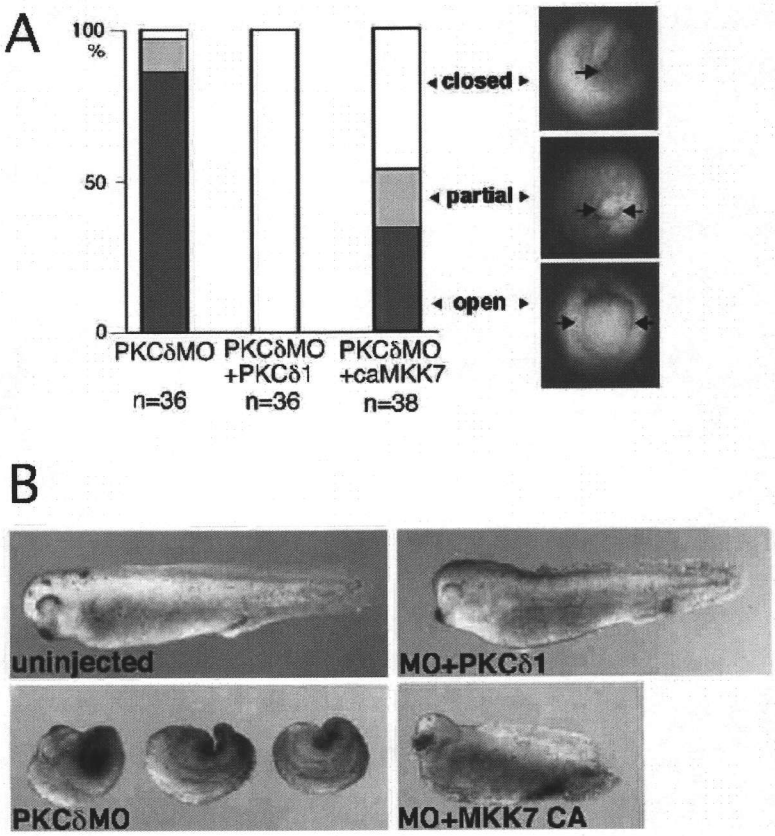

Figure 3-12. PKC δ loss-of-function is rescued by overexpression of active MKK7.

(A) Twenty nanograms of *PKC δ* MO were coinjected with 1 ng of *PKC δ 1*, and 200 pg of constitutively active (CA) *MKK7*. The closure of the blastopore of injected embryos was compared at stage 14, when the blastopore in the control embryos was completely closed. *PKC δ* MO blocked gastrulation movement. *PKC δ* mRNA rescued the phenotype completely, and CA *MKK7* mRNA partially rescued it. (B) At the tadpole stage, embryos coinjected with CA *MKK7* showed a partially rescued phenotype.

suggests that PKCδ may act not only through the JNK pathway, but in other pathways as well.

PKCδ is not essential for the canonical Wnt pathway

I next tested whether PKCδ plays a role in the canonical Wnt pathway. It is known that the ectopic expression of Wnt8 induces secondary axis formation (Smith and Harland 1991) and marker genes such as *siamois* and *Xnr3* by activating the canonical Wnt pathway in *Xenopus* embryos (Brannon and Kimelman 1996; Carnac et al. 1996; McKendry et al. 1997). When *Wnt8* was coinjected with *PKCδ ΔC* or *PKCδ* MO, the induction of *siamois* and *Xnr3* by Wnt8 was not inhibited (Fig. 3-13). Furthermore, the secondary axis formation by Wnt8 was not inhibited by the loss of PKCδ function (data not shown). Therefore, although PKCδ is required for the Wnt/JNK pathway, it may not be necessary for the canonical Wnt pathway, which is independent of the membrane relocalization of Dsh (Yang-Snyder et al. 1996; Axelrod et al. 1998; Moriguchi et al. 1999; Rothbacher et al. 2000).

PKCδ regulates the cell shape and intercalative behavior of the mesodermal cells during convergent extension movements

During convergent extension of the mesoderm, cells are polarized and aligned mediolaterally, then intercalated. To test whether PKCδ is required in this process, the convergent extension in DMZ explants was observed microscopically. The procedure was basically according to Wallingford et al. (2000). *PKCδ* MO, Rhodamine dextran, and mRNA for Venus (a YFP variant; Nagai et al. 2002) fused with the membrane localization signal of K-ras (mb-Venus) were coinjected into one of the two dorsal blastomeres at the four-cell stage. As a control, mb-Venus mRNA alone was injected into the other dorsal blastomere (Fig. 3-14 A). At the gastrula stage, dorsal marginal zone explants were cultured on a cover glass coated with FN. These explants adhered to the surface, and subsequently, convergent extension movements occurred in the mesoderm. In the absence

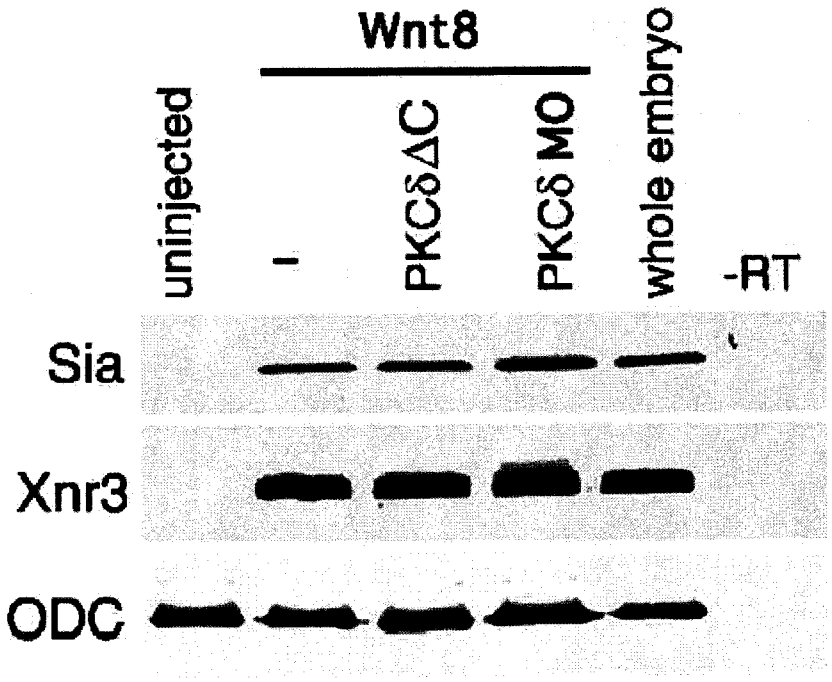

Figure 3-13. PKC δ is not essential for the canonical Wnt pathway.

To test whether PKC δ is essential for the canonical Wnt pathway, 100 pg of *PKC δ Δ C* or 20 ng of *PKC δ* MO was coinjected with 10 pg of *Wnt8* into *Xenopus* embryos. *PKC δ Δ C* and *PKC δ* MO did not inhibit the induction of *siamois* or *Xnr3*, indicating that PKC δ does not affect the canonical Wnt signaling pathway.

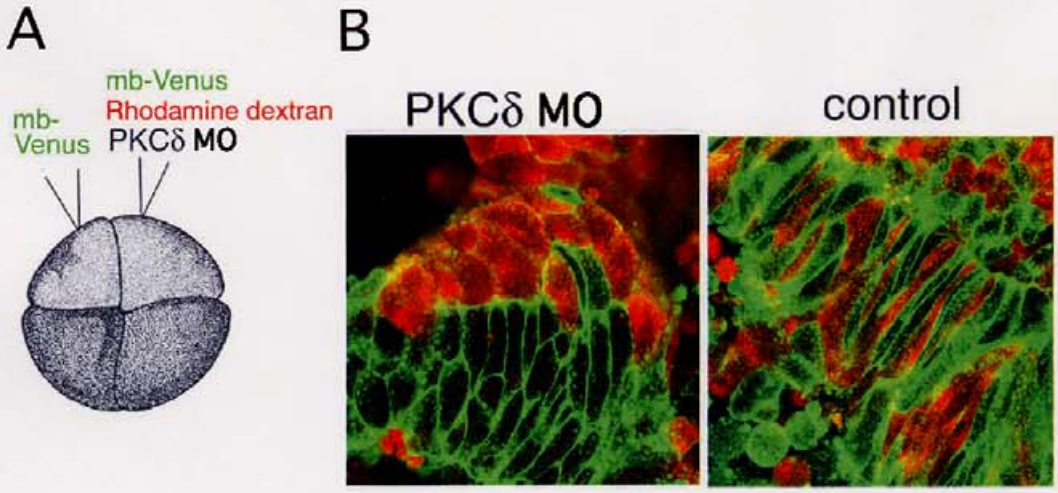

Figure 3-14. PKC δ is required for convergent extension movements.

(A) *PKC δ* MO, Rhodamine dextran, and mRNA for Venus fused with a membrane localization signal (mb-Venus) were coinjected into one of the two dorsal blastomeres at the four-cell stage. As a control, mb-Venus mRNA alone was injected into the other dorsal blastomere of the same embryo. (B) At the gastrula stage, dorsal marginal zone (DMZ) explants were cut and cultured on a cover glass coated with fibronectin, and the convergent extension movements were observed by laser-scanning confocal microscopy.

of *PKCδ* MO, red cells and non-red cells intercalated (Fig. 3-14 B). In the *PKCδ* MO-injected explants, the non-red cells, which were assumed to lack the MO, were polarized and showed convergent extension movements. In contrast, the red cells (MO-injected cells) were round-shaped, were not polarized, and did not participate in the intercalation, even when they were adjacent to the intercalating cells. Thus, this inhibition by *PKCδ* MOs appeared to be cell-autonomous. These results indicated that PKCδ is essential for the cell polarization during convergent extension movements. To investigate the subcellular localization of PKCδ and Dsh in the dorsal mesodermal cells, I expressed these proteins tagged with Venus. Interestingly, these were accumulated around the tips of the elongated cells (Fig. 3-15). However, Dsh lacking the DEP domain, which is known to play an important role in the tissue polarity (Axelrod et al. 1998) was almost uniformly distributed (DshΔDEP). In addition, Rac tagged with Venus is also localized in the same region. The finding that Rac forms a complex with Dsh (Habas et al. 2003) suggests that Rac may be recruited by Dsh. The process of convergent extension movements includes cell shape change and cell movements, suggesting that the regulation of actin polymerization may be crucial for the process. It is known that the Arp2/3 complex is a key component of the assembly of actin filaments and the cell motility (Suetsugu et al. 2002; Weaver et al. 2003). Thus, I tested the localization of the Arp3 tagged with Venus in these cells. As shown in Figure 3-15, Arp3 is also localized around the tips of these cells. In addition, Rac and Arp3 were localized almost uniformly on the membrane or the cortical region when *PKCδ* MO was injected. The Arp2/3 complex may be recruited by PKCδ and its downstream PCP signaling and may regulate the cell polarity, bipolar protrusive activity, and cell motility in these cells.

Loss of function of G proteins affected morphogenesis in the early *Xenopus* embryos.

Because Frizzled is a seven-transmembrane receptor, it is very likely to couple with heterotrimeric G proteins. Thus, I next attempted to examine the role of G proteins in the regulation of gastrulation. To

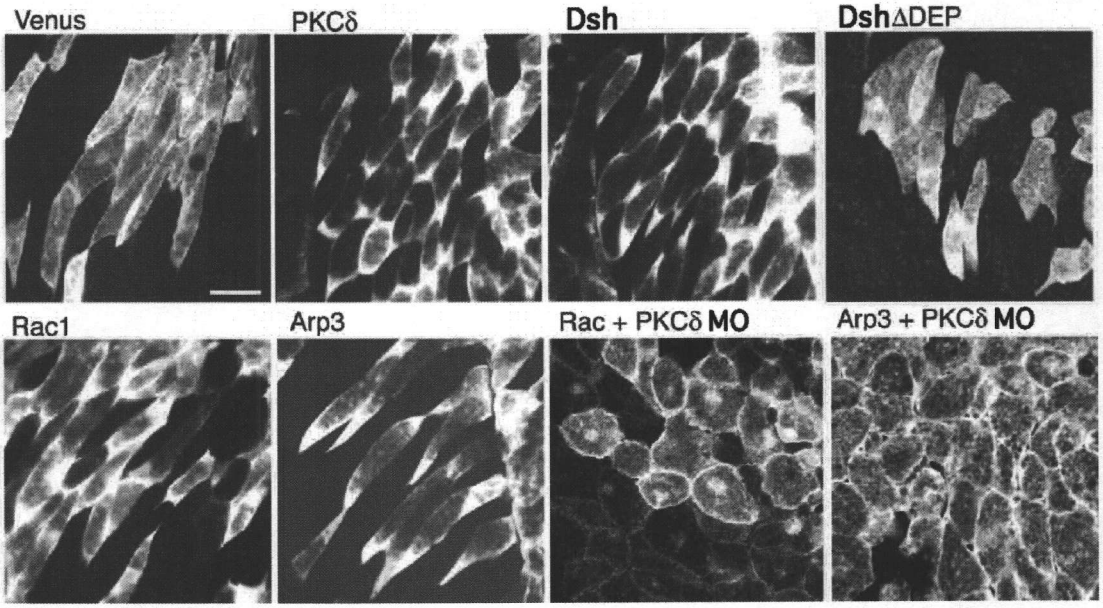

Figure 3-15. Dsh, PKC δ and actin regulators are localized at bipolor cell tips during convergent extension movements.

The indicated cDNAs were fused to Venus and expressed in the dorsal mesodermal cells. DMZ explants were cultured and observed as described above. Bar, 50 μm.

investigate G protein functions, I designed several MOs targeted to 5'-UTR of each G protein mRNA. These MOs specifically reduced expression of each G proteins (Fig. 3-16). To examine the knockdown phenotypes of these G proteins on convergent extension movements, these MOs injected into dorsal blastomeres of 4 cell embryos. As a result, two MO injected embryos showed morphogenetic defects. *Gi1* MO-injected embryos showed a short-trunk phenotype, and *G11* MO-induced a gastrulation defect (Fig. 3-17). These MOs also inhibited the mesodermal elongation (Fig. 3-18). These phenotypes are also caused by the inhibition of the PCP pathway, suggesting that these MOs affect this signaling pathway.

Both G_{i1} and G_{11} are required for the plasma membrane localization of Dsh

Next, I tested whether these MO affect the localization of Dsh. It has been shown that Dsh is localized in the cytoplasm of the animal cap cells, and Fz7 expression translocates it to the plasma membrane. In this case, Dsh is uniformly distributed on the plasma membrane. I found that co-expression of Fz7 and Wnt11 localized Dsh to patch-like structures on the plasma membrane (Fig. 3-19). Physiological significance of Dsh localization has not yet been clear. But when the Fz7 and Wnt11 were co-expressed, a downstream signals were more strongly activated, suggesting that the patch-like localization may represent strong activation of the PCP pathway. Both *Gi1* and *G11* MOs inhibited the membrane localization of Dsh by Fz7 expression and its patch-like accumulation by co-expression of Fz7 and Wnt11 (Fig. 3-19). The function of G_{i1} and G_{11} are required for Dsh translocation by the Wnt signaling. *G11* MO inhibited Dsh translocation more effectively than *Gi1* MO. This is consistent with the MO-injected phenotypes that *G11* MO seemed to more severely inhibit convergent extension than *Gi1* MO. These results strongly suggested that these G proteins might mediate the signal from Fz7 to Dsh.

G_{i1} and G_{11} play different roles to translocate Dishevelled in response to Wnt signaling.

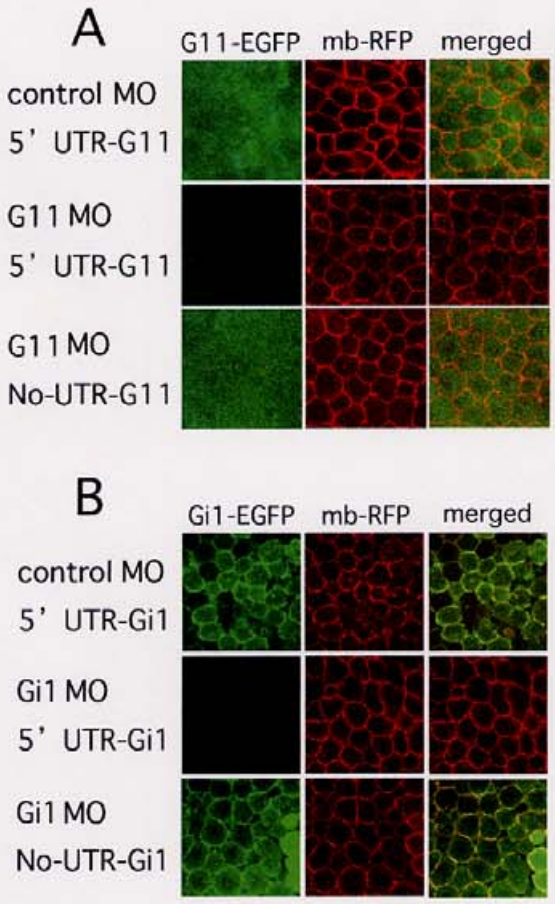

Figure 3-16. G_{11} MO and G_{i1} MO specifically and effectively inhibit protein synthesis

(A) G_{11} MO specifically inhibited protein synthesis. *G_{11}-EGFP* fusion genes with or without the 5'-untranslated region (UTR) containing the MO-targeting sequence were constructed (*UTR-G_{11}* and *no-UTR-G_{11}*, respectively). When they were expressed in animal cap explants, both *UTR-* and *no-UTR-G_{11}* were detected at the same levels and were located on the plasma membrane. G_{11} MO inhibited the translation of *UTR-G_{11}*, but not of *no-UTR-G_{11}*. The protein expression of an unrelated control, mb-RFP was not affected. This result led us to expect that G_{11} MO could specifically and effectively inhibit the endogenous G_{11} protein synthesis. (B) G_{11} MO also inhibited G_{11} protein synthesis.

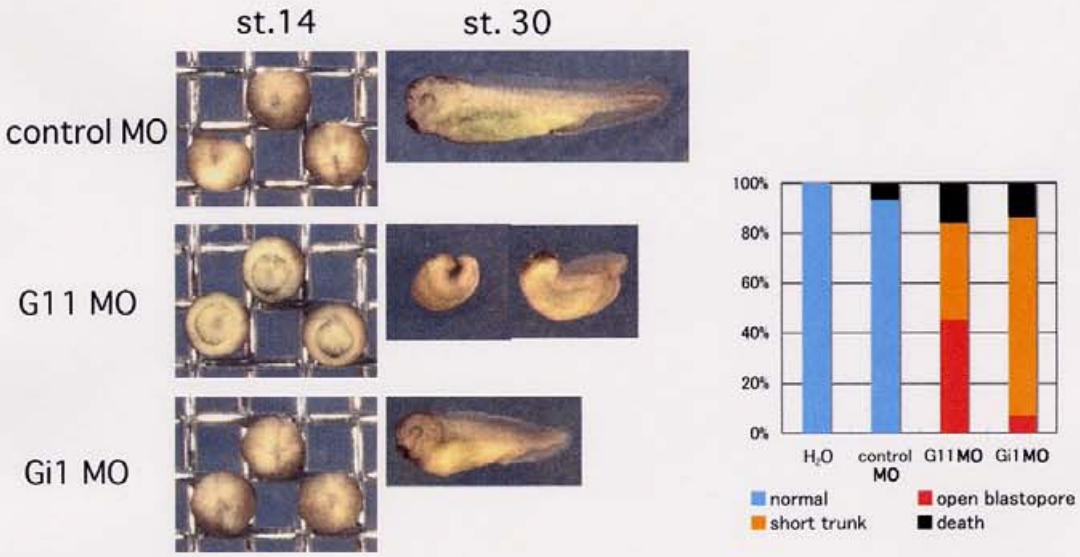

Figure 3-17. G_{11} or G_{i1} loss of function caused morphogenetic defects.

G_{11} or *G_{i1}* MO injected embryos showed morphogenetic defect. *G_{11}* MO (5 pmol) injected embryos induced the gastrulation defect. *G_{i1}* MO (10 pmol) injected embryos mainly showed a short-trunk phenotype, (Fig. 3-17).

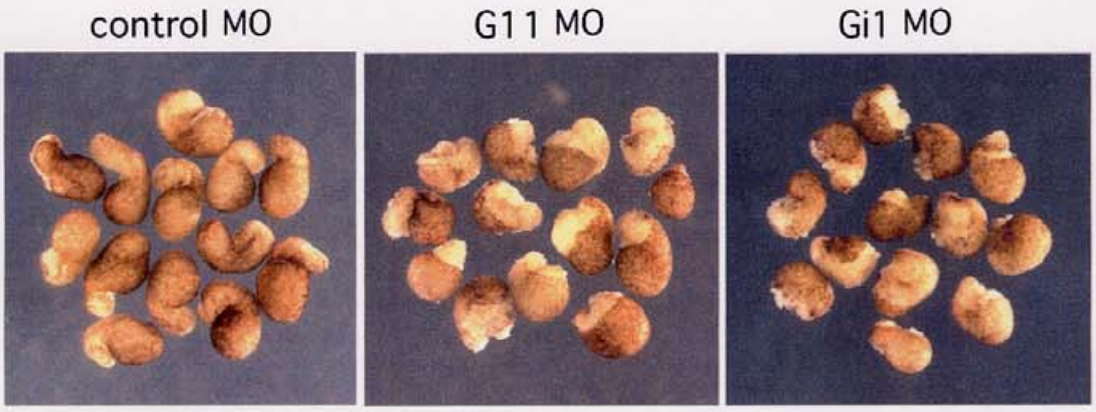

Figure 3-18. Both G_{11} and G_{i1} MO inhibited mesodermal elongation.

Both G_{11} MO (5pmol) and G_{i1} MO (10pmol) inhibited the elongation of dorsal marginal zone explants. Each MOs were injected into the two dorsal blastomeres of four-cell embryos.

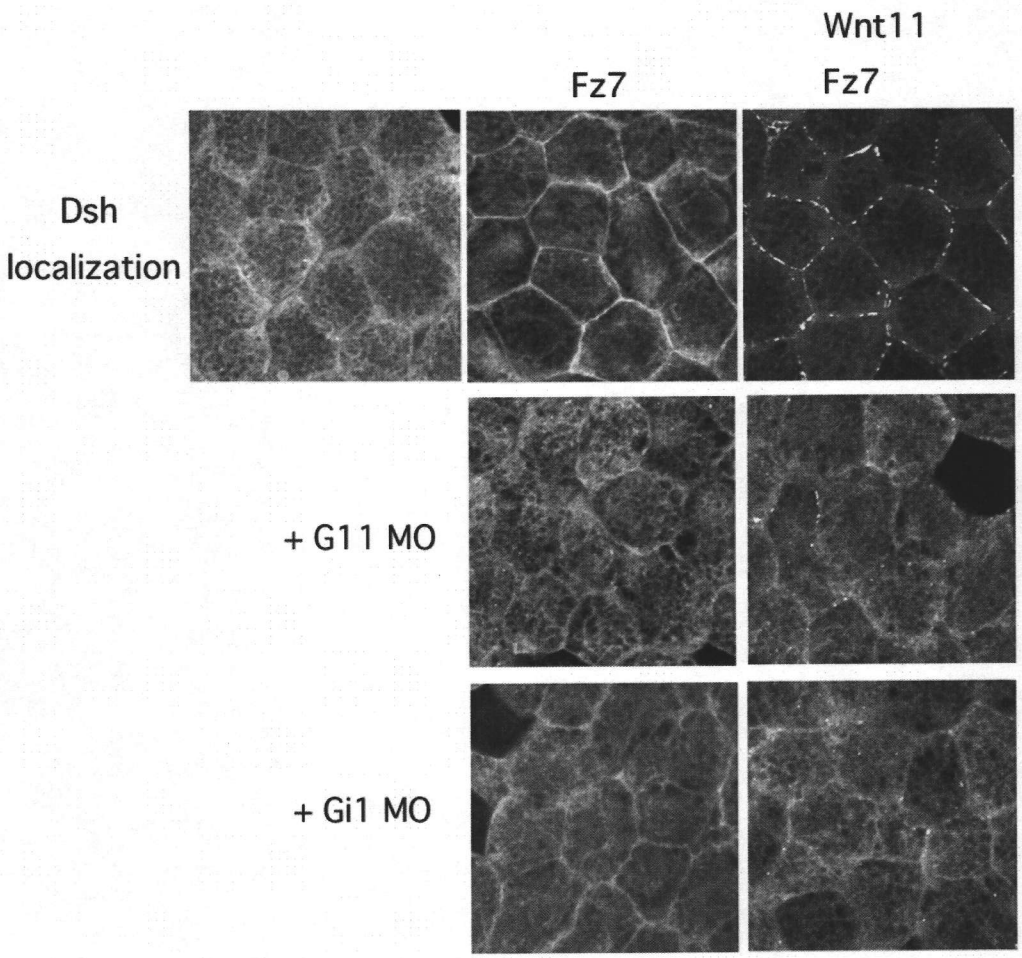

Figure 3-19. Both G_{11} and G_{i1} MO inhibited the Dsh translocation in response to Wnt signaling.

Myc-tagged Dsh translocates in response to Wnt signaling. *myc-Dsh* mRNA (100 pg) was coinjected with *Fz7* mRNA (100 pg) and *Wnt11* (100 pg) into animal pole of 2-cell embryos. At st.9, these animal caps were isolated and immunostained with anti-myc monoclonal primary antibody (9E10), and Cy3 conjugated anti-mouse IgG monoclonal secondary antibody. After immunostaining, confocal microscopic analyses were carried out.

To examine whether G proteins might be required for the PCP pathway, I tested two assays, (1) hyperphosphorylation of Dsh and (2) the promotion of the protrusive activity by the PCP pathway in the animal cap cells. In these assays, I obtained similar results. *G_{11}* MO reduced hyperphosphorylation of Dsh and inhibited the protrusive activity by Wnt signaling, whereas *G_{i1}* MO did not (Fig. 3-20,21). Considering together with the above result that G_{11} inhibited Dsh translocation more effectively than G_{i1}, G_{11} may play a major role in mediating the signal from Fz7 to Dsh.

It is known that G protein coupled receptors can transduce signals even when the receptor is directly fused with the G protein α subunit that couples with the receptor (Milligan., 2002). To examine whether Fz7 directly fused to G_{11} or G_{i1} may also transduce the signal, I constructed genes encoding Fz7 C-terminally fused with G_{11}, $_{i1}$, $_{i2}$ and $_{i3}$. When these are expressed in the animal cap cells, Fz-G_{i1} translocated Dsh to the plasma membrane, whereas Fz-G_{i2} and $_{i3}$ did not have such activities. These results strongly suggest that Fz7 can directly and specifically couple with G_{i1}. G_{i2} and G_{i3} fused to Fz7 may inhibit the activity by interfering the interaction between Frizzled and endogenous G proteins. It is unexpected that Fz-G_{11} had no activity. One explanation is that G_{11} might have to dissociate from Fz7 to signal to the downstream. Further experiments are definitely required.

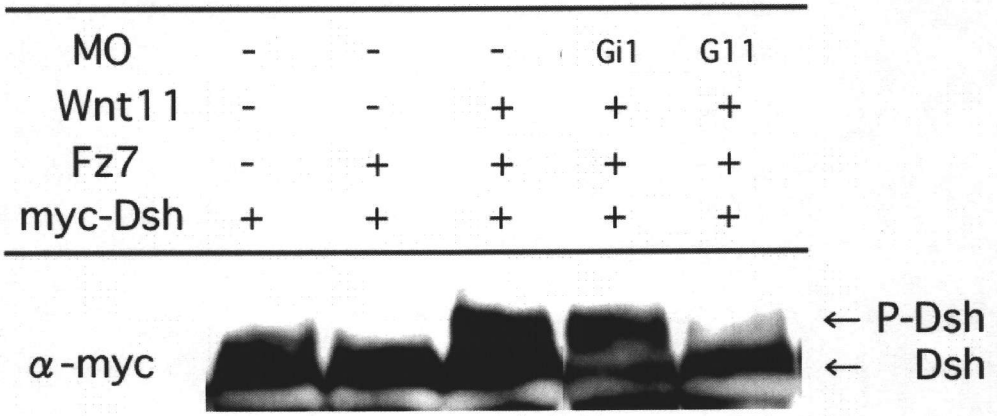

Figure 3-20. *G11* MO inhibited hyperphosphorylation of Dsh but not *Gi1* MO. 100 pg mRNA of *myc-Dsh*, *Fz7*, and *Wnt11* were coinjected with *G11* or *Gi1* MO into animal pole of 2-cell embryos. Animal cap explants were isolated from st.9 embryos. Lysates from isolated explants were run in SDS-PAGE and analyzed by Western blotting.

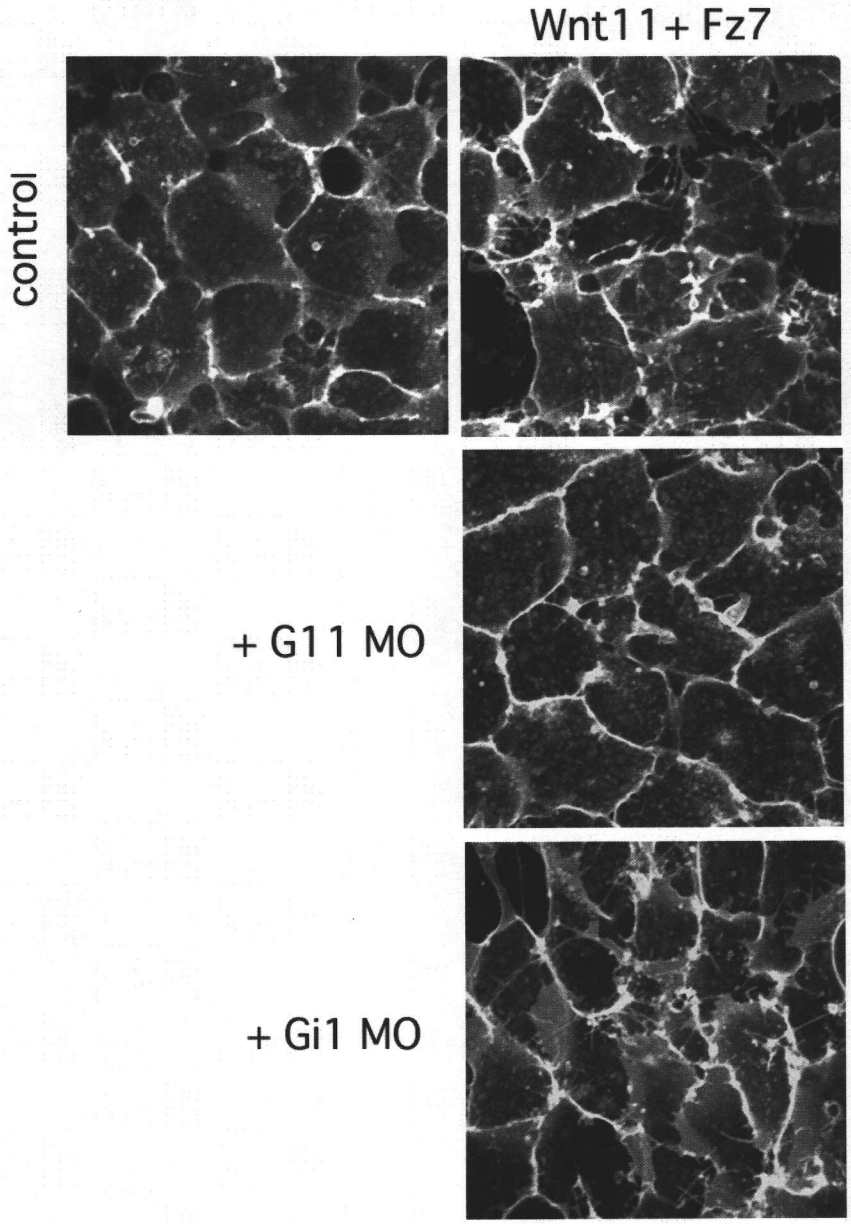

Figure 3-21. G_{11} **function is required for the protrusive activity regulated by Wnt signaling.**

Wnt11 and *Fz7* mRNAs (100 pg each) were coexpressed in animal cap explants with mb-Venus. The coexpression of *Wnt11* and *Fz7* promoted the protrusive activity. 5 pmol of G_{11} MO and inhibited it but not G_{i1} MO (15 pmol).

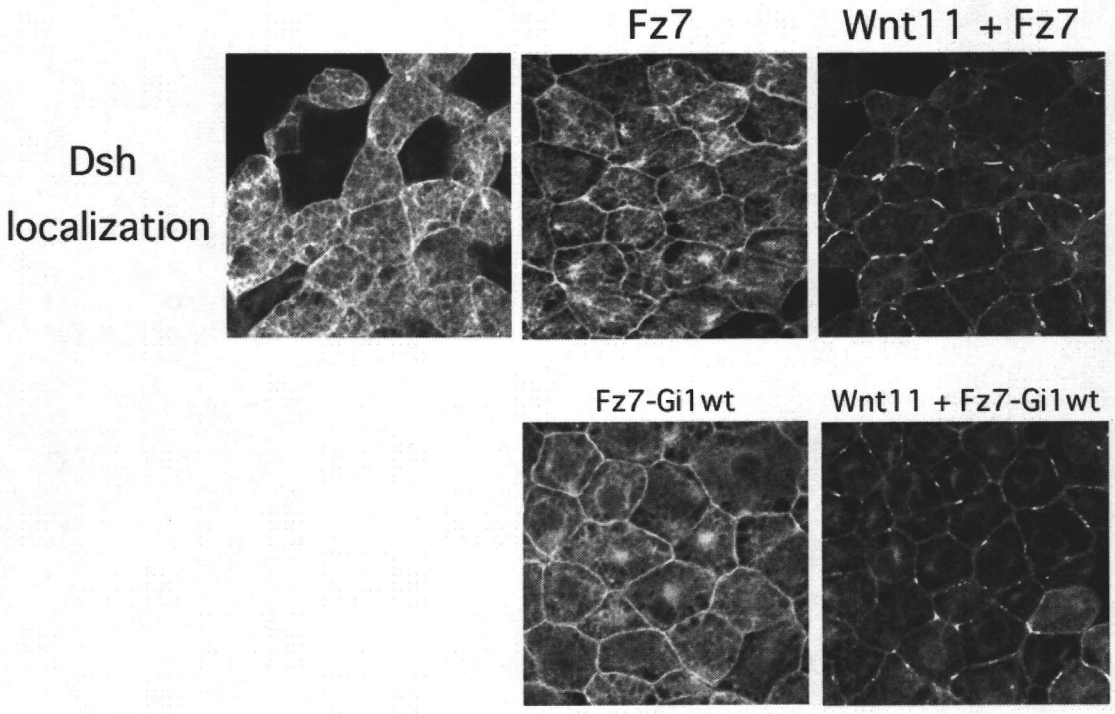

Figure 3-22. Wild type G_{i1} fused to Fz7 translocate Dsh to the plasma membrane.

Myc-Dsh, Wnt11 and *Fz7* mRNAs (100 pg each) were coexpressed in animal cap explants. Dsh translocated by Wnt signaling. *Wild type G_{i1} fused Fz7* mRNA injected animal explants also showed Dsh translocation. *G_{i1} fused Fz7* mRNA did not affect Dsh localization (data not shown).

DISCUSSION

PKC δ and G protein α subunits G_{i1} and G_{11} are involved in the PCP pathway

The membrane localization of Dsh is thought to be an important step for Dsh activation in the PCP pathway (Moriguchi et al. 1999; Rothbacher et al. 2000; Wallingford et al. 2000; Axelrod 2001). However, the molecular mechanism of this process has not been clarified. In the present study, I have investigated roles of PKC δ, G_{11} and G_{i1} in the PCP pathway, and revealed that PKC δ and G_{11} are essential for the translocation and activation of Dsh in the PCP pathway, based on the following findings. The loss of PKC δ or G_{11} function inhibited the membrane localization and hyperphosphory -lation of Dsh. In addition, PKC δ physically interacts with Dsh. In the many biological systems, G_{11} is known as an activator for phospholipase C β (PLC β). Taking all together, I propose the following model of the Dsh activation mechanism in the PCP pathway. In the absence of Wnt/Fz7 signaling, Dsh and PKC δ are localized in the cytoplasm. When the receptor is activated, G_{11} that couples with Fz7 is activated. The activated G_{11} activates PLC β that produces, diacylglycerol (DAG). Then, PKC δ, which has a DAG-binding C1 domain, is translocated and recruits Dsh to the membrane. PKC δ may be activated by DAG, resulting in the hyperphosphorylation of Dsh. The membrane localization and/or phosphorylation of Dsh activate the downstream JNK pathway. This model is also supported by the result that PMA was sufficient to recruit Dsh when PKC δ was overexpressed. G_{i1} seems to play a minor role in the PCP pathway, because *G_{i1}* MO did not inhibited the hyperphosphorylation of Dsh and the protrusive activity induced by the PCP pathway so effectively as *G_{11}* MO. However, *G_{i1}* MO significantly inhibited convergent extension in DMZ explants, and the elongation of the body axis. Furthermore, *Fz-G_{i1}* fusion gene can translocate Dsh to the plasma membrane. These results suggested that Fz7 couples to G_{i1} as well as G_{11}. It is known that PLC β is activated not only by $G_{q/11}$, but also by Gβ/γ (Rhee 2001). It would be an interesting

possibility that G β/γ complex dissociated from G_{i1} might activate PLC β. This may explain that Fz-G_{i1} can activate the downstream signal because G β/γ complex is dissociated from the α subunit to activate PLC β. Both G_{11} and G_{i1} may be required for full activation of PLC β. More experimental data are necessary to examine this possibility.

Is G protein/PLC β signaling sufficient for the activation of the PCP pathway? It is known that the C-terminal cytoplasmic region of Fz is required for activation of downstream signaling pathway. Previous data showed that Dsh interact with this domain directly (Wong et al., 2003). I demonstrated that this region is also required for Dsh translocation by PCP signaling (data not shown). These data suggest, that not only G proteins, but also the other signaling components such as the C-terminal domain of Fz7 may be required for the activation of the PCP signaling.

Role of the PCP pathway in convergent extension

Convergent extension is comprised of several steps involving changes in cell morphology and movements. As convergent extension begins, cells extend lamellipodia in random directions. The cells are then polarized and become narrow along the mediolateral direction, followed by the intercalation of these cells. Dsh function is required for this regulation of cell polarity (Wallingford et al., 2000). I clearly showed that *PKC* δ MO-injected cells were not polarized, nor did they participate in the intercalation, indicating that PKC δ is essential for controlling cell polarity and the change in cell shape during convergent extension movements. Interestingly, PKC δ and Dsh tagged with Venus were localized around the tips of the elongated cells. In addition, Rac and Arp3 are also localized in the same regions. It is known that these regions have a lamellipodial protrusive activity. Arp3 is one of Arp2/3 complex components, which is a key regulator of the actin polymerization in lamellipodial protrusion of membranes (Suetsugu et al.. 2002; Weaver et al., 2003). The Arp2/3 complex is also known to be regulated by Rac (Suetsugu et al., 2002). It is strongly suggested that the proper localization of Rac, Arp3, and other cytoskeletal regulators may be important for cell elongation and intercalative movements. Without

PKC δ function, cells were round-shaped, and Rac and Arp3 did not localize in the specific region. This suggests that PKC δ, Dsh and the downstream PCP signaling may be required for the localization of such machinery, including the Arp2/3 complex.

CHAPTER 4

CONCLUSION AND SUGGESTIONS FOR FUTURE STUDIES

In this thesis, I focused on cytoskeletal regulation and mechanism of signal transduction of the PCP pathway in *Xenopus*. In Chapter2, I analyzed MARCKS function in cytoskeletal regulation during gastrulation. MARCKS was required for control of cell morphology, motility, adhesion, protrusive activity, and cortical actin formation in embryonic cells. I also demonstrate that the PCP pathway promotes the formation of lamellipodia- and filopodia-like protrusions and that MARCKS is necessary for this activity. These findings show that MARCKS regulates the cortical actin formation that is requisite for dynamic morphogenetic movements regulated by the PCP signaling.

The molecular mechanism to signal from Fz7 to Dsh has not yet been elucidated in the PCP pathway. In chapter 3, I described the identification of three identified molecules essential for this signal transduction. One of these is PKC δ. PKC has been implicated in the Wnt signaling pathway, however, its molecular role is poorly understood. Loss of function experiments revealed that PKC δ was essential for convergent extension during gastrulation. The others are heterotrimeric G protein α subunits G_{i1} and G_{11}. I examined the relationship between these molecules and the Dsh localization in *Xenopus* animal cap explant. Loss of function of these molecules inhibited both Dsh translocation in response to Wnt11/Fz7 signaling in animal cap cells and convergent extension movements in embryos. These G proteins and PKC δ are, thus, essential for signaling from Fz7 to Dsh in the PCP pathway.

The PCP pathway plays crucial roles in convergent extension movements during *Xenopus* gastrulation. A number of PCP related proteins (Wnt, Frizzled, Dishevelled, Strabismus, Prickle, Flamingo, Diego...) have been identified and analyzed. However, the essential aspects in the PCP pathway were obscured mainly due to the following three points.

1. Downstream signaling of Dsh is unclear. It is known that RhoGTPases and JNK are signaling components in the PCP pathway. But their roles in the PCP pathway are unclear.
2. The information about subcellular localizations of PCP signaling components is insufficient. To show when, where, and how PCP molecules function in the cells will give us useful information.
3. The signal transduction from the receptor to Dsh is still unclear. To understand the PCP signaling, it is important to clarify the coupling specificity between Frizzled receptors and G proteins. Furthermore, how the heterotrimeric G proteins are activated and transduce downstream signaling is also an interesting question. Some molecules such as RGS (regulator of G protein signaling) and AGS (activator of G protein signaling) that are known as G protein signaling regulators in other systems may be implicated in the regulation of the PCP pathway.

I think that these points are critical for further understanding the PCP pathway. For the first point, I think that protein binding assay between Dsh and other molecules may be interesting. But it will be difficult because the signaling pathway downstream of Dsh may be divergent. For the second point, timelapse microscopic analyses may be necessary. For example, imaging of the vesicle transport, the membrane trafficking, the cell shape change, and the molecular localization change may be of significance for further understanding of the PCP pathway. Finally, for the third point, G proteins may play previously unknown roles in the PCP pathway. For example, it is known that G proteins regulate asymmetric cell division, spindle positioning and assembly. These data suggest that G proteins coupled with Frizzleds may also play divergent roles and regulate other biological process.

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